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THE HELIOTROPISM OF ONCHIDIUM: A PROBLEM IN THE ANALYSIS OF ANIMAL CONDUCT.

By W. J. CROZIER and L. B. AREY.

(*Contributions from the Bermuda Biological Station for Research, No. 108, and from the Physiological Laboratory, College of Medicine, University of Illinois.*)

(Received for publication, July 21, 1919.)

I.

Onchidium floridanum is a small naked pulmonate which lives in holes and crevices of the shore between tides. A description of the bionomics of this mollusk we shall present more fully in another connection,[1] omitting here those aspects not bearing directly upon the most curious puzzle propounded by its behavior, which in brief is this: under natural conditions the movements of *Onchidium* take place in the light, but without reference to photic control; yet when the animal is tested apart from its natural surroundings it is found always to be negatively phototropic.

In view of this apparent incompatibility, in reality very deep seated, one is led to inquire: Is the behavior of *Onchidium* an instance where "unnatural conditions" imposed in phototropism experiments vitiate attempted analytic explanation of normal activities and more especially what, fundamentally, is the *sense* of the state of affairs we have outlined, in terms of adaptation?

II.

1. Phototropism.—Exploration of the dome-shaped dorsum of the mantle of *Onchidium* with a light-beam 0.3 mm. in diameter and of moderate intensity showed that the movements of the mollusk, in the laboratory could be directed at will by even as small an illumi-

[1] A preliminary statement of certain of the results, utilized for different purposes than in the present connection, is found in the papers: Arey and Crozier (1918) and Crozier and Arey (1919*a*).

nated area as 0.3 sq. mm. To accomplish this result it was necessary to stimulate the anterior end of the mantle, which is more irritable, photically, than other regions of the mantle. The animal contracts on the non-stimulated side, and creeps away from the source of activation. The whole mantle dorsum is photosensitive, local areas puckering sharply into depressed pockets, secondarily extending as furrows across the animal's back, when one spot is illuminated. The anterior end of the foot, and the oral lappets, are more sensitive in this respect.

The tentacles, which in some *Onchidia* carry terminal eyes, are non-reactive to light, nor are the orienting movements of the animal interfered with in any way by the removal of the tentacles.

It therefore appears that although, as we have ascertained, this species of *Onchidium* does not possess differentiated mantle eyes (Semper, 1877; Stantschinsky, 1908), the physiological precursors, or perhaps the remnants, of such structures are actively functional in *Onchidium floridanum*.

Onchidium is quite unresponsive to increase of light intensity as such, but is very promptly and precisely oriented by incident light having a horizontal intensity component. There is found at all times and without exception a precise negative phototropism. That such orientation is in no way determined by "changes of intensity" is adequately demonstrated by the fact that these animals are at the same time conspicuously reactive to shading. When the light falling upon an *Onchidium* in air is suddenly decreased, the tentacles are forcibly withdrawn beneath the mantle, the head is retracted, locomotion stops, and the mantle is pressed into contact with the substratum. The distribution of this mode of irritability coincides with that evidenced in responses initiated by illumination. As with *Holothuria*, *Chiton*, and some other forms, the nature of the photic orientation of *Onchidium* is profoundly inconsistent with the differential sensitivity exhibited by the same animal (Crozier and Arey, 1918).

2. In the Field.—Onchidium lives during high-tide in "nests," cavities in the rock containing a number of individuals, from which the mollusks at low water emerge to feed upon exposed shore surfaces. The individuals emanating from any one nest return simultaneously to that nest before the tide rises again (Arey and Crozier, 1918).

The natural wanderings of the *Onchidia* while feeding upon the rocks take place without reference to the nature of the sunshine, whether brilliant or dull, and bear no relation whatsoever, at any time, to the direction of the incident light. The *Onchidia* creep out from their sheltering cavities only during daylight hours, however, and never at night, no matter how bright the moon.

In the laboratory, as far as our numerous experiments have shown, *Onchidium* moves away from a source of light under any conditions of temperature (15–32°), dryness (under water, or in air), and light (regardless of intensity) compatible with its active existence; whereas, on its natural substratum the same individual may creep directly into the horizontal rays of the setting sun, or away from them, with indifference. This state of affairs is well exemplified by the fact that, if an *Onchidium* is picked up from its natural substratum and a glass plate slipped between it and the rock, the animal orients immediately away from the sun, and with machine-like precision. Mere disturbance is not responsible for this phenomenon, because an equivalent handling of the mollusk, followed by its replacement on the rock, does not lead to the exhibition of negative heliotropism (provided the animal is restored to the rock within a certain radius of its "nest").

Similar results are obtained if an *Onchidium* creeping on the shore is shaded from the sun and then reilluminated from a new direction by light reflected from a mirror. Momentary "hesitation" may succeed such illumination, but the creature's path is not materially influenced.

3. Inhibition of Heliotropism.—We would emphasize the point that the natural movements of *Onchidium* cannot be viewed as "contrary to the dictates of the animal's heliotropism;" but that *during the creeping of the mollusk on the rock surface immediately surrounding its "home" this heliotropism is completely inhibited.* Proof of the correctness of this contention is seen in these facts, each of which has been verified upon a number of occasions.

(*a*) Removal of the oral lappets of an *Onchidium*, organs which normally are in constant contact with the substratum, or their anesthetization by $MgSO_4$, obliterates the normal directed homing of the animal; at the same time its negative heliotropism becomes a dominant factor in the control of creeping.

(*b*) An *Onchidium* removed to a strange section of shore moved about in a manner largely or entirely directed by the illumination. The same is true of any *Onchidium* which has been kept in an aquarium for 24 hours and is then returned to its native scene.

(*c*) In favorable instances, injection of *Onchidia* with strychnine (0.1 to 0.2 cc. of 1 per cent strychnine hydrosulfate), while not producing more than a moderate temporary contraction, succeeded by normal creeping, leaves the snail at the mercy of its heliotropism, although the animal may not have been handled at all, the injecting needle being simply inserted in its back and discharged.

These facts make it evident that in nature, as far as directed progression is concerned, the heliotropism of *Onchidium* is simply inhibited, or suffers central block, probably owing to those guiding impulses originating in the substratum and mediated through the oral lappets. Strychnine, while not necessarily "converting inhibition into excitation" (Cushny, 1919; Arey and Crozier, 1919), lowers the central threshold of solar activation impulses.[2]

It is improbable, furthermore, that the diurnal rhythmicity of *Onchidium's* appearance from its concealed rock cavities has any relation to heliotropism. The tidal rhythm of appearance is such, as we have learned by long observation, that even (as in summer) though two periods of low water may occur during daylight hours, the *Onchidium* colonies creep out to feed but once in the 24 hours. On the other hand, especially in winter,[3] several days may pass before a tidal period occurs in good daylight; the *Onchidia* then may make no appearance for several days. The brightness or dullness of the day has probably, as far as we can detect, no influence on emergence.

III.

With reference to the adaptation of habits to scene of life, the other activities of *Onchidium* are no less curious than its directed creepings ("homing") on the shore. It might be said that it is to the best interests

[2] "Reversals" of an analagous sort, but normally determined through feeding activities, are possibly involved in "homing" movements; this matter we shall treat separately.

[3] *O. floridanum* does not hibernate, as some other species appear to do (Hirasaka, 1912) in colder climates.

of this animal to come out into the sunlight; it must do so in order to obtain food; therefore, it disregards the dictates of its negative phototropism. This notion is sufficiently disposed of in the foregoing section; the negative heliotropism of *Onchidium* is not "disregarded" normally, it is centrally inhibited by other impulses competing for the nervous control of the body musculature. The further question remains: What is the rôle of the heliotropism of *Onchidium*? The mere existence of this type of response is sufficiently accounted for by the composition of the animal and the structural disposition of its parts. We have to inquire if this heliotropism is ever of "use."

Note in the first place our finding that the movements of an *Onchidium* on a rock substratum remote from its own "nest" are largely, and, in many instances, exclusively determined by the position of the sun. Might it not then be supposed that if an *Onchidium* should accidentally suffer displacement from its usual surroundings, its negative orientation by light would force it to enter some crevice or rock cavity, thus securing shelter and perhaps membership in a new colony? But the facts are different. The natural displacement of an *Onchidium* in this way is probably of very rare occurrence; these snails possess a well disposed system of repugnatorial glands (Crozier and Arey, 1919*a*), which makes such an event unlikely. Much more serious, however, is the fact that the animal withdraws sharply when shaded, as already stated. Furthermore, numerous instances we have carefully watched have yielded not one good case in which an *Onchidium* would enter a strange "nest" and remain there for more than a few minutes near the entrance (Arey and Crozier, 1918); they always crept out and away. These two kinds of response make it improbable that a "lost" *Onchidium* would seek refuge in a strange depression, and those which were purposely displaced in this way remained in fact creeping about on the rock until washed off by the rising tide.

IV.

We must refuse, then, to admit any obvious adaptive significance inhering in the photic behavior of *Onchidium*. Loeb has pointed out (1916) that positive heliotropism occurs in several arthropods "which have no opportunity to make use of it." Instances of this

kind have drawn from some writers the curious comment that "these reactions are non-adaptive only under artificial conditions" (Mast, 1911), with the further assumption that "the reactions were inherited from ancestors in which they were adaptive." The normal inhibition of heliotropism in *Onchidium* is adaptive, if you like, but the further difficulty of finding some adaptive excuse for the existence of heliotropism at all remains all the more insoluble. The behavior of *Onchidium* is more curious than that of the crustaceans cited by Loeb, for there is physical opportunity to "make use" of their photic sensitivity, since they are exposed to light. Whether the mechanism for such a response is merely an "inheritance" from ancestors in which it was adaptive, one need not say, but the mechanism is fully present, though seemingly not used, and of obscure, if any, functional value—as far as concerns the movements of *Onchidium* on the rock surfaces.

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THE COMBINATION OF ENZYME AND SUBSTRATE.

I. A Method for the Quantitative Determination of Pepsin.

II. The Effect of the Hydrogen Ion Concentration.

By JOHN H. NORTHROP.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, July 7, 1919.)

I. A Method for the Quantitative Determination of Pepsin.

Considerable experimental evidence has been obtained by various authors to show that enzymes are removed from their solutions by insoluble substances.[1,2] Pepsin has been especially studied from this view-point. Dauwe[3] showed that this property of removing pepsin from its solution was not common to all substances and was connected in some way with the digestive action of the enzyme. He was also able to show that the size of the particles, at least in the case of egg albumin, was without any marked effect on the amount of pepsin removed.

Abderhalden[4] and his coworkers state that pepsin is removed almost completely from its solution by insoluble proteins on which it acts and they consider that this plays an important rôle in the kinetics of the reaction. These results were partially confirmed by Leary and Shieb.[5]

[1] For the literature see Bayliss, W. M., The nature of enzyme action, London, 3rd edition, 1914.

[2] Nelson, J. M., and Griffin, E. G., *J. Am. chem. Soc.*, 1916, xxxviii, 1111.

[3] Dauwe, F., *Beitr. chem. Physiol. u. Path.*, 1905, vi, 426. This paper reviews the earlier work.

[4] Abderhalden, E., and Steinbeck, E., *Z. physiol. Chem.*, 1910, lxviii, 293. Abderhalden, E., and Strauch, F. W., *ibid.*, 1911, lxxi, 315. Abderhalden, E., and Wachsmuth, F., *ibid.*, 339. Abderhalden, E., and Friedel, F., *ibid.*, 449. Abderhalden. E., and Kramm, F., *ibid.*, 1912, lxxvii, 462.

[5] Leary, J. T., and Shieb, S. H., *J. Biol. Chem.*, 1916–17, xxviii, 393.

Bayliss[1] attaches considerable importance to the combination of enzyme and substrate and considers it an essential point in the theory of enzyme action. Van Slyke and Cullen[6] were able to formulate the kinetics of enzyme action on the basis of the law of mass action by assuming the existence of a compound between enzyme and substrate.

The study of this combination, however, has not furnished any quantitative experimental data as to the nature of the reaction or the influence of various factors on it—due largely to the difficulty of determining quantitatively the amount of enzyme.

It seemed important, therefore, to obtain quantitative experimental data on this subject. In order to do this it was necessary to have a convenient and accurate method for the determination of pepsin. It was found that the change in the conductivity during the digestion of egg albumin by pepsin afforded such a method. Sjöqvist[7] found that there were marked changes in the conductivity during pepsin digestion. His results were confirmed with the exception that the change was found not to follow the actual rate of digestion. It can be used therefore only as an empirical method for the determination of pepsin and not for the study of the kinetics of the reaction, as was done by Sjöqvist. The reason for this divergence is probably due to the fact that the change in conductivity is due to two causes; (1) the liberation of free acid (carboxyl) groups which increase the conductivity, and (2) the liberation of free amino groups which bind some of the free acid and so decrease the conductivity. This explanation is borne out by the following facts.

With dilute solutions of egg albumin containing strong acetic acid (pH 2.3) there is a regular small increase in the conductivity which nearly parallels the increase in free amino groups as followed by the increase in amino nitrogen. This is due to the fact that, owing to the very large excess of free undissociated acid present, the ions which are removed from solution by combination with the free amino groups are replaced by the dissociation of more acid, and so kept nearly constant. The slight increase in conductivity is therefore due to the liberation of free carboxyl groups. In strong acid solu-

[6] Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1914, xix, 141.

[7] Sjöqvist, J., *Skandin. Arch. Physiol.*, 1893–95, v, 277.

tions, however, as hydrochloric, there is a rapid decrease in the conductivity—due to the removal of acid ions by combination with the liberated amino groups. This change is so much larger than the increase due to the acid groups set free by the protein that the increase in conductivity due to the latter is more than compensated. It has already been shown[8] that the actual rate of digestion is approximately the same in all acids at the same reaction so that the differences in the changes in the conductivity cannot be ascribed to differences in the rate of digestion.

It was found that the maximum change occurs in strong solutions of egg albumin titrated to pH 2.6 with hydrochloric acid. This solution was therefore used. The conductivity was followed by means of the apparatus described by Taylor and Acree.[9] The electrodes were of the dipping type and were immersed in the solution in order to make a reading. It was found that the percentage change in conductivity was constant for a given quantity of pepsin, irrespective of the absolute value of the original conductivity. The readings and figures are therefore given in terms of the increase in the scale readings on the bridge, which for small readings are equivalent to the percentage change. The measurements were carried out as follows.

25 cc. of a 3 per cent egg albumin solution were pipetted into a series of large "Non-sol" test-tubes and suspended in a water bath at 37° ± 0.02°. The electrodes were immersed in the solution and 1 cc. of the pepsin solution was added. The external resistance was then set so as to give a bridge reading of 500; *i.e.*, the middle of the bridge. The change in conductivity was now followed by the bridge readings. These figures are related to the actual change in resistance of the solution by means of the formula $\frac{X}{R} = \frac{A}{1{,}000 - A}$ where X = resistance of the solution, R is the external resistance, and A is the bridge reading.

The figures given in Table I are the increase in the value of A and are very nearly proportional to the percentage increase in the resistance.

[8] Northrop, J. H., *J. Gen. Physiol.*, 1918–19, i, 607.

[9] Taylor, W. A., and Acree, S. F., *J. Am. Chem. Soc.*, 1916, xxxviii, 2396.

Readings were taken at intervals so as to give points corresponding to changes of 2 to 4 units of the bridge reading (which could be easily read to 0.25 units). These points were then plotted on a large scale by means of a flexible "spline" and weights, so that the curves were

TABLE I.

Change in Conductivity of Solution of Egg Albumin with Varying Amounts of Pepsin.

25 cc. of egg albumin solution, pH 2.6. *R* 320.
Temperature 37° ± 0.02°C. *A* at beginning 500.

Relative amount of pepsin added in volumes of 1 cc.

100		50		25		12.5	
Time.	Increase in scale reading.	Time.	Increase in scale reading.	Time.	Increase in scale reading.	Time.	Increase in scale reading.
min.		*min.*		*min.*		*min.*	
3	4.0	4	3.0	3	1.5	7	2.2
8	8.5	13	7.8	17	6.0	9	2.7
14	12.0	23	11.0	28	8.0	26	5.2
18	14.5	39	16.0	46	11.5	34	6.7
31	21.0	51	19.5	60	13.5	67	9.2
43	26.0	88	26.5	93	18.5	102	13.2
83	38.0						

Change.		Time necessary to cause change.		
Increase in scale reading.	Time.			
	min.	*min.*	*min.*	*min.*
5 to 10	6.0	11.8	22.4	44.0
10 to 15	8.1	14.4	32.0	

Interval.	Relative amount of pepsin calculated.			
5 to 10	(100)	51	27	13.6
10 to 15	(100)	56	25	
Average.....	(100)	53	26	13.6

30 to 50 cm. long. The time necessary to cause a given change was then interpolated from these curves. In this way errors in individual readings were smoothed out and, as the curves are regular, accurate values for the time necessary to cause a given change could be ob-

tained. Since there is always some uncertainty about the zero reading the interval from 0 to 5 was omitted and the time to cause a change of from 5 to 10 and from 10 to 15 units was taken. The values obtained in this way were then averaged and the result taken as proportional to the reciprocal of the amount of pepsin present. By comparing this figure with the corresponding one from a control containing a standard amount of pepsin, the relative quantity of pepsin present could be determined, since it was found that the time

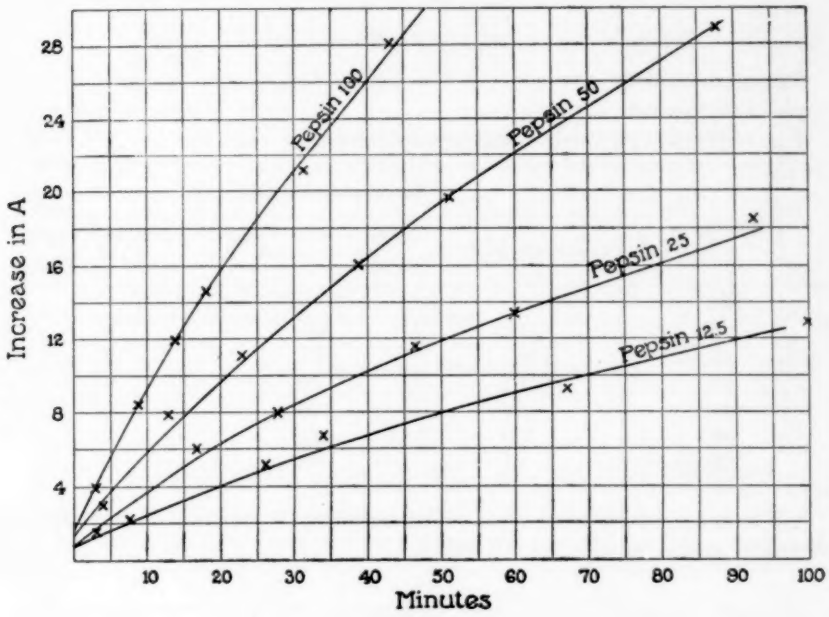

FIG. 1.

necessary to cause any given change was inversely proportional to the amount of pepsin present. That is, double the quantity of pepsin requires half the time, etc. (Arrhenius' "QT" rule).[10]

Table I and Fig. 1 give the results with a series of solutions containing different amounts of pepsin. It will be seen that the amount of pepsin can be determined with an accuracy of ± 2 to 3 per cent,

[10] Arrenhius, S., Quantitative laws in biological chemistry, London, 1915.

and further that ten to fifteen determinations can be made in the course of 1 to 2 hours depending on the amount of pepsin present.

It was now possible to determine quantitatively the amount of pepsin removed from solution by various substances.

Table II shows the results of such a series with coagulated and dried egg albumin. The control experiments show that the decrease in the amount of pepsin is not due to the destruction of the pepsin on standing or to the retarding effect of the products of reaction.

TABLE II.

Change in Concentration of Pepsin in Various Solutions.

Temperature 25°C.
pH of all solutions, 2.5.
1 cc. of solution pipetted off and pepsin estimated at time noted.

Time.	Relative amount of pepsin per cc.		
	20 cc. of pepsin solution.	20 cc. of pepsin solution + 1 gm. of egg albumin in solution.	20 cc. of pepsin solution + 1 gm. of coagulated egg albumin.
min.			
1	(100)	101	95
10	101	101	84
20	94	96	82
40	95	100	86
80	100	128*	80
160	97	98	86

* This is an experimental error. The curve was irregular and gave widely divergent results for different intervals.

A series of experiments was now made with various substances. The results are summarized in Table III. It is obvious that the removal of the pepsin is not purely a matter of surface but that it is dependent in some way on the substance itself.[11]

This fact is brought out more strongly in the experiments summarized in Table IV and Fig. 2, where coagulated egg albumin of different sized particles was used. It is evident that the equilibrium

[11] The author does not doubt the existence of adsorption or concentration in the surface layer in the sense of Willard Gibbs. This phenomenon, however, is evidently of subordinate importance here.

reached is independent of the size of the particles and therefore of their surface. These experiments were repeated under slightly different conditions several times—always with the same result. This would indicate that the process is either one of solution, in which

TABLE III.

Removal of Pepsin from Solution by Different Substances.

10 cc. of pepsin solution, pH 2.5, + 0.5 gm. of substances noted. Allowed to stand 10 min. at 25°C. and pepsin estimated in 1 cc. of solution.

Substance.	Relative quantity of pepsin per cc.
Control; pepsin solution alone	100
Starch	98
$CaSO_4$	86
Agar	103
Kaolin	100
Blood charcoal	15
Casein	70
" (coagulated, dried, and ground to 40 mesh)	74
" (extracted with boiling alcohol for 24 hrs.)	60
" C (charred at 150°C.)	90
Egg albumin (coagulated, dried with acetone, and finely powdered)	10

TABLE IV.

Effect of Size of Particles of Egg Albumin, Coagulated, Dried, and Ground.

2.0 gm. in 20 cc. of pepsin solution titrated to pH 2.5 + HCl.
1.0 cc. pipetted off and analyzed for pepsin at time noted.

Time.	Size of particles.	
	Through 10 mesh but not through 20 mesh.	Through 80 mesh.
	Relative amount of pepsin per cc.	
min.		
1		46
2	98	21
4	93	30
8	72	12
30	32	10
60	12	14
120	10	11

case the law of partition coefficients should be found to hold, or of chemical combination, in which case the law of mass action should apply. Preliminary experiments indicate that the process follows the law of partition coefficients.

It may be objected that the effective surface is not the actual surface of the particles but some fine interior structure which is the same in all. The particles, however, swell in acid and appear translucent and homogeneous. It would seem that any interior surfaces must be of nearly intermolecular dimensions. In this case, of course, all chemical phenomena may be considered "surface" phenomena.

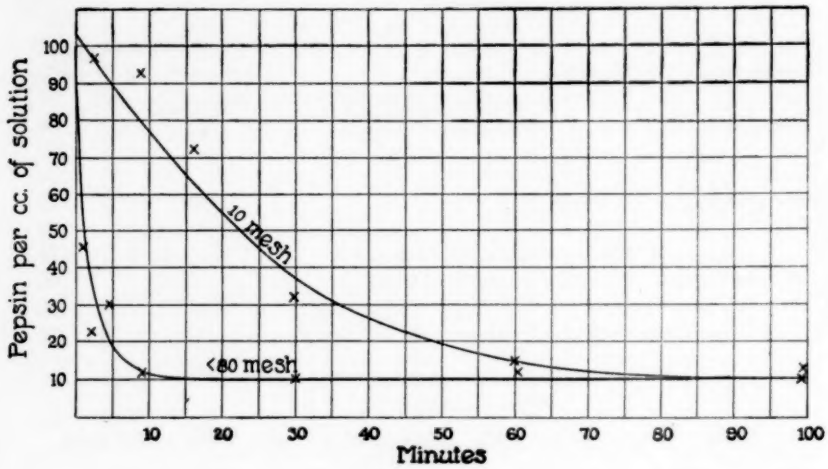

FIG. 2.

II. The Effect of the Hydrogen Ion Concentration.

Inasmuch as the activity of pepsin is dependent to a large extent on the hydrogen ion concentration it appeared probable that the combination of pepsin with its substrate would be a function of the same variable. Table V and Fig. 3 show the results of a series of experiments made at different hydrogen ion concentrations. There is a decided optimum for the combination of pepsin with its substrate corresponding (within the limits of error) to the optimum for digestion. These experiments were repeated with casein with approximately the same results. The optimum zone for the digestion of proteins

by pepsin therefore is due to the fact that at this degree of acidity more pepsin combines with the protein than in either a more or less acid solution. Van Slyke and Zacharias,[12] from a study of the constants of their equation for the action of urease, decided that the hydrogen ion concentration affected the *rate* of combination of the

TABLE V.

Effect of Reaction of Solution on Combination of Pepsin and Coagulated Egg Albumin.

Experiment A.

Temperature 25°C.

0.5 gm. of egg albumin suspended in 10 cc. of HCl of increasing strength. 1.0 cc. of strong pepsin solution added. Tube shaken, allowed to stand 1 min., and clear liquid pipetted off. pH measured (by gas chain) in part of this sample. 5.0 cc. of remainder brought to same reaction in all tubes by addition of a few drops of strong HCl. All brought to same volume with water and pepsin estimated in 1 cc.

Experiment B.

Same as A, but allowed to stand 2 min.

pH of solution.	Relative amount of pepsin per cc.		No albumin. (Control.)	Relative amount of pepsin combined.	
	Experiment A.	Experiment B.		Experiment A.	Experiment B.
0.88	77		100	23	
1.00		79			21
1.24	69			31	
1.3		68			32
2.08	60		100	40	
2.2		50			50
2.75	63			37	
2.8		47			53
3.5	64			36	
3.9		68			32
4.4	77	80		23	20
4.9	100	86	100	0	14
5.7	100	94	100	0	6

enzyme and substrate. In the case of pepsin, however, it is not the *rate* of combination but the *amount* which is influenced. This is shown by the fact that little or no pepsin is removed from its solution by its substrate at a reaction of 5.0, no matter how long they are

[12] Van Slyke, D. D., and Zacharias, G., *J. Biol. Chem.*, 1914, xix, 181.

left in contact. It seems probable that this is due to a change in the condition of the protein rather than to a change in the enzyme, since, according to Ringer,[13] the optimum reaction is different for different proteins.

The simplest explanation of the above facts would seem to be that the quantity of ionized protein present determines the amount of pepsin which combines with the protein, and hence also determines the rate of digestion. Some direct evidence confirming this hypothesis has been obtained and will be discussed fully later.

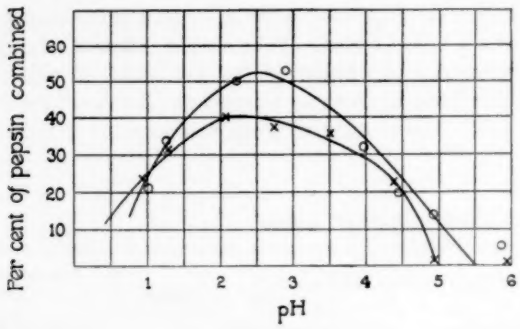

FIG. 3.

SUMMARY.

1. A quantitative method for the determination of pepsin is described depending on the change in conductivity of a digesting egg albumin solution.

2. The combination of pepsin with an insoluble substrate has been followed by this method.

3. The amount of pepsin removed from solution by a given weight of substrate is independent of the size of the particles of the substrate.

4. There is an optimum zone of hydrogen ion concentration for the combination of enzyme and substrate corresponding to the optimum for digestion.

5. It is suggested that the pepsin combines largely or entirely with the ionized protein.

[13] Ringer, W. E., *Kolloid-Z.*, 1916, xix, 253.

LABYRINTH AND EQUILIBRIUM.

I. A Comparison of the Effect of Removal of the Otolith Organs and of the Semicircular Canals.

By S. S. MAXWELL.

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(Received for publication, September 16, 1919.)

INTRODUCTION.

According to the conceptions of Mach and Breuer we must distinguish two kinds of equilibrial functions in the ear; the one, dynamic through which movements of rotation are perceived, and the other, static by which is produced a definite orientation or sensation of position in relation to the lines of gravitational force. It was suggested that the dynamic function is performed by the sensory structures of the semicircular canals, the static by the otolith organs of the vestibule. Although this view has been widely accepted the literature of the subject is full of contradictions and the experimental evidence is far from satisfactory.

Results of experiments on the labyrinth on which I have been engaged for a long time show that no sharp differentiation exists between the functions of the otolith organ of the utriculus and the ampullæ of the semicircular canals. I have found on the one hand that a labyrinth from which the ampullæ have been removed without injury to the vestibular portions possesses both dynamic and static functions, and that on the other hand a labyrinth from which the otolith organs have been removed without injury to the ampullæ retains both static and dynamic functions.

The experiments reported in this article have been made on dogfish of the genera *Mustelus* and *Squalus*. The advantages of the dogfish as an experimental animal have often been pointed out. Among these are the large size of the various parts of the labyrinth, the

clearness with which operations may be performed through the transparent cartilage of the skull, and the fact that bleeding can be almost wholly avoided and that the fish recovers readily from the shock of the operation.

In my experiments it has been necessary to use as indices of results the well known compensatory movements which occur on rotation, the righting reaction, and the general state of equilibrium. The compensatory movements of the eyes and fins of the dogfish, as first described by Loeb,[1] are remarkably constant. I have made especial use of the eye movements which may be described briefly as follows: if a dogfish is held in its normal resting position, belly down, and longitudinal body axis horizontal, the eyes assume a characteristic resting position, symmetrical to the body. This position of the body I shall refer to as normal and the position of the eyes as the primary position. If the animal is rotated to the right around its longitudinal axis, the eyes make complementary movements of such character that they tend to preserve their original position in space; that is, the right eye is elevated and the left eye is depressed. If the rotation is around the transverse axis, head inclined downward, both eyes appear to rotate on their axes by a wheel-like movement so that the anterior pole of each eye is elevated and the posterior pole is depressed. If the animal is rotated backward around its transverse axis both eyes make a compensatory wheel-like forward rotation. If the animal is rotated about an obliquely placed horizontal axis the compensatory movement is a resultant of the effects which would be produced by the rotations around the two axes of reference. These compensatory movements are made during the rotation, but if the abnormal body position is retained, the complementary position of the eyes is also retained. It is seen, of course, that as long as the animal is kept in the abnormal position there is a constant force; namely, the force of gravity, acting upon its body elements in an unaccustomed direction.

If the animal is held in the normal position and the body is then rotated around its dorsoventral axis, both eyes turn by a conjugate movement in the opposite direction; that is, if the head is swung to the right both eyes turn to the left, and if the head is swung to the

[1] Loeb, J., Ueber Geotropismus bei Thieren, *Arch. ges. Physiol.*, 1891, xlix, 175.

left both eyes look to the right. If in these rotations the new position is retained, the new position of the eyes is not retained; they return to their primary position. It is seen at once that in the new position there is no altered relation to the lines of gravitational force.

Removal of the Ampullæ.

The ampullæ of the anterior vertical and the horizontal canals open into the utriculus so near the macula acustica of the latter that most experimenters seem to have had difficulty in destroying the one set of structures without injury to the other. Lee[2] and Lyon[3] each speak of destruction of the ampullæ, but both seem to have relied on section of the nerve branches. By section of the nerve branches, however, they arrived at exactly opposite and fundamentally contradictory results.

After considerable practice I have developed a special technique by which the ampullæ of any or all of the canals may be removed with a minimum of injury and shock to the animal and with results which admit of no uncertainty. A flap of skin is loosened and turned back exposing the appropriate portion of the skull. A thin surface layer of the skull is sliced off with the attachment of some of the neck musculature, thus making visible the parts of the labyrinth through the transparent cranial cartilage. The membranous canal is exposed at a distance not too great from its ampullar enlargement. With a fine pointed pair of curved forceps the membranous canal is grasped as closely as possible to the ampulla and the canal with its ampulla is extracted by a sudden movement, a light quick jerk. Success in this operation depends mainly upon the choice of forceps with the proper curve which bite at the very point, and upon acquiring the knack of removal of the canal by a suitable movement. A too sudden pull will usually break off the canal external to the ampulla, and too slow a movement frequently drags and injures portions of the vestibular structures which it is desired to leave unharmed. When

[2] Lee, F. S., A study of the sense of equilibrium in fishes, *J. Physiol.*, 1893, xv, 311.

[3] Lyon, E. P., A contribution to the comparative physiology of compensatory motions, *Am. J. Physiol.*, 1899–1900, iii, 86.

one has once acquired the knack of this operation the results become absolutely clear. The ampullæ can be extracted one after another with certainty and exactness. In sectioning the nerves one may cut too much or too little; the fiber bundles are scattered, and certainty is impossible. The attempted destruction of the ampullæ *in situ* cannot by any means have the exactness of their complete removal. In many of my earlier experiments I had the ampullæ pasted on a blank leaf of my note book when I wrote down on the same page the results of their extirpation. Under these conditions there can be no doubt as to the correctness of the results. In the summer of 1919, I repeated and extended these experiments at the Marine Biological Laboratory, and on account of the contradictions of previous workers I took occasion to have the experiments witnessed by a number of physiologists and zoologists.

I had found[4] that removal of the ampullæ of the four vertical canals had little or no effect on the compensatory eye movements resulting from the rotation around the longitudinal and transverse axes. In order, however, that there could remain no possible functioning of the semicircular canals I have in a long series of animals removed all six ampullæ with uniform results.

A dogfish from which all six ampullæ have been removed shows definitely the following reactions. (1) Compensatory movements of the eyes and fins occur on rotation around a longitudinal axis; *e.g.*, on rotation to the right, the right eye goes up and the left eye goes down. This position of the eyes is retained as long as the abnormal body position is continued. (2) Compensatory movements of eyes and fins occur on rotation around the transverse axis; *e.g.*, when the animal is tilted head downward the eyes make the characteristic wheel-like backward rotation. (3) Compensation is absent on rotation around the dorsoventral axis. (4) The animal swims in a manner differing but little from the normal. (5) The righting reaction takes place promptly and vigorously; if the animal is placed belly up in water it turns over at once.

As a sample experiment I quote *verbatim* the following from my notes.

[4] Maxwell, S. S., Experiments on the functions of the internal ear, *Univ. California Pub. in Physiol.*, 1910–15, iv, 1.

"July 14, 1919. Dogfish 5. 10.00 a.m. All six ampullæ removed. Compensatory movements prompt on rotation around longitudinal and transverse axes; none on rotation in horizontal plane. Animal rights itself perfectly in water. Eyelids sewed together to exclude retinal stimuli, and animal put into deep tank; righting perfect.

2.00 p.m. Animal rather weak but rights itself promptly when turned over in water; swims rather wobbly; turned completely over once when excited by other dogfish; I have seen a normal dogfish do this under similar circumstances.

July 15, 9.00 a.m. Animal very weak; rests on bottom of tank in normal position. Rights itself but may swim one or two turns belly up before getting over. Opened stitches in eyelids. No compensatory movements of eyes.

July 16, 9.30 a.m. Animal moribund. Killed for autopsy. Considerable blood clot in each vestibule."

The above experiment shows a possible source of the confusion in the reports of previous investigators. Had I assumed that on account of shock effects observations made on the day of operation would be unreliable, and had I waited until the following day to make my observations, it would have appeared that loss of the ampullæ abolishes compensatory movements, which is manifestly not true. When immediately following the destruction of an organ a function is clearly retained it is indisputable proof that at least that organ is not the only one which can perform the function. Observations made on July 15, on Dogfish 5, might have favored the statement that destruction of the ampullæ of the semicircular canals abolishes compensatory movements of the eyes, but the observations of July 14 clearly show such a conclusion to be wrong.

In the attempts to determine the rôle of the various sense organs in the geotropic reactions of the dogfish, it has long been recognized that retinal stimuli play a part. Lyon[3] and Parker[5] excluded visual stimuli by section of the optic nerve. I have accomplished the same result by the less radical operation of sewing the eyelids together, when equilibrium and the righting reactions were under consideration, and by placing a black, opaque disk on the cornea over the region of the pupil when eye movements were to be studied. Other methods of blinding were also used. I can affirm with complete assurance

[5] Parker, G. H., Influence of the eyes, ears, and other allied sense organs on the movements of the dogfish, *Mustelus canis* (Mitchill), *Bull. Bureau of Fisheries*, 1909, xxix, 43.

that the compensatory motions described in the case of animals from which all the ampullæ have been removed occur also when activity of the retina has been excluded.

It must also be noted that the dogfish, like most animals which rest on the bottom and are not merely suspended in the water, manifests very strong contact reactions. A vigorous specimen which has been blinded and which has had as far as possible all the end organs of the eighth nerve destroyed will almost always be found belly down when at rest. Such a fish may swim indifferently back or belly up, but when it comes to rest the position is a fair index of the general state of the animal. When an investigator affirms that his specimen came to rest indifferently in any position, he has given good incidental evidence as to the animal's physical condition. In stating that a dogfish deprived of its six ampullæ makes normal righting reactions I have not been unmindful of these facts, but have taken care to exclude the possibility of contact stimuli.

Although it can be proved that after the loss of all the ampullæ, with exclusion at the same time of retinal and contact stimuli, the dogfish makes normal compensatory movements of the eyes and fins to rotations in all vertical planes, it is necessary to note that there are some differences between this and a normal animal.

The following seem to be fairly constant results. (1) The compensatory movements of the eyes, though prompt, are noticeably slower than in the uninjured animal. Compensatory movements due to visual stimuli alone are so much slower, requiring several seconds or even minutes, that no difficulty is experienced in distinguishing these from reflexes of labyrinthine origin. (2) If seized while in the water the animal strongly resists the attempt to turn it back downward. One feels, however, that the resistance is neither as prompt nor as strong as in a normal animal. (3) In swimming there is more or less evident a slight tendency to sway from side to side around the longitudinal axis, like a boat insufficiently ballasted.

These three conditions are less noticeable in vigorous specimens; they become very marked in weakened individuals. They can perhaps all be accounted for by a lowering of muscle tonus. It is important to note that, as I shall show later, precisely the same complex of conditions can be brought about through a totally different operation.

Removal of the Otoliths.

In the dogfish the otoliths are of soft, friable, calcareous material. In the sacculus there is a large otolith spread over the main macula acustica and a smaller mass on the lagena. These are so situated that their removal can be accomplished with little injury and the operation is relatively easy. For the otolith of the utriculus the case is very different. This otolith lies in the recessus utriculi so close to the openings of the ampullæ of the anterior vertical and the horizontal canals that it requires some skill and much practice to remove it without injury to the ampullæ. If, however, after opening the vestibule by removing a portion of the cartilaginous roof, the utricular wall is slit open with a very sharp microdissection knife, the otolith material may be washed out by the careful use of a fine pointed pipette. In a similar way the saccule may be slit open and its otoliths removed. No operation was considered successful unless it was found at postmortem examination that no otolith material remained. For reasons to be stated in another paper it was considered important not only to avoid injury of the ampullæ but also to reduce the injury of the utriculus to a minimum.

After removal of all the otoliths from both ears in successful cases the following results are seen. (1) Compensatory movements of the eyes are made in the regular way to rotations about all three body axes, longitudinal, transverse, and dorsoventral. If the animal is rotated around a longitudinal or transverse axis and held in the abnormal position the compensatory position of the eyes is retained, but when the rotation is around the dorsoventral axis the eyes make the compensatory movement and then return to the primary position. These movements appear to differ from those in the normal animal only in being slightly slower. (2) The animal swims in normal orientation and maintains its equilibrium in the water, but its swimming, like that of the fish without ampullæ, is likely to be accompanied by a rocking movement; this rocking or swaying is less apparent in vigorous specimens. (3) If turned belly up in the water, the fish rights itself promptly; in doing so, however, it sometimes overcompensates and turns almost or completely over.

It will be seen that these results are strikingly similar to those

produced by loss of the ampullæ. It is especially noticeable that there is the same apparent slight slowing of the reactions and the same indication of lowering of muscle tonus in general. In one important respect, however, the result of this operation differs from that of removal of the ampullæ; namely, the compensatory movement to rotation about the dorsoventral axis is retained. It should be stated that in these observations due care was taken to eliminate retinal and contact reactions.

Parker[6] removed the saccular otolith by way of an opening in the roof of the mouth. He found that the loss of this otolith alone produced no noticeable effect on the equilibrium or the righting reactions of the dogfish, nor did there appear to be any loss of tonus. I have removed this otolith many times by the method I have described above. Its loss does not alter or weaken any of the compensatory movements; it does not disturb the equilibrium or the righting reaction, nor is the muscle tonus affected to any noticeable degree.

Removal of Both Ampullæ and Otoliths.

I may say at once that in accordance with the findings stated in the last section the presence or absence of the large otolith of the saccule is without influence on the equilibrium reactions and it may be disregarded. If, however, the utricular otolith has been successfully removed and the condition described in a previous section has been attained, namely the retention of compensatory movements to rotations in all planes, the righting reaction, and the maintenance of equilibrium, the consequent removal of the six ampullæ produces at once a profound alteration. The condition of a dogfish deprived of the utricular otolith and the six ampullæ may be stated in the following way. (1) No compensatory movements are made on rotation around any axis whatever. This statement may be modified by saying that in some cases a slow and slight tendency to compensation, requiring many seconds or even minutes for its completion, may be seen. No one familiar with the reactions of the animal would ever confuse this with a labyrinthine reflex. (2) The animal shows no tendency to maintain bodily equilibrium; it swims indifferently back or belly upward. A weak specimen may also come to rest on its

side or back, but a vigorous specimen usually rights itself on the bottom of the tank. In other words the geotropic reactions of the animal are definitely and completely lost; the stereotropic reaction is retained.

CONCLUSION.

The results of my experiments show that the assumption of a sharp differentiation of function between the otolith-bearing, vestibular portions of the labyrinth and the semicircular canals is not justified by the facts. Between the effects of extirpation of the one and of the other set of structures there is more resemblance than contrast. They certainly reenforce each other, for the reactions produced by either one alone are always slower and less vigorous than when both sets of organs are intact. It would not, however, be safe to affirm that the functions are identical. In one respect a difference is apparent; namely, in the response to rotation in a horizontal plane. If the ampullæ are uninjured, compensatory movements occur when the animal is rotated around its dorsoventral axis. I have never seen this reaction in the absence of the ampullæ of the horizontal canals.

SUMMARY.

1. A dogfish from which all six ampullæ have been removed maintains its equilibrium; the righting reactions occur promptly; compensatory movements of the eyes occur in response to rotations in all planes except the horizontal; the compensatory position of the eyes is retained if the animal is held in an abnormal position. Both the static and dynamic functions of equilibrium continue, therefore, after complete removal of all the semicircular canals and all the ampullæ.

2. After complete removal of the otoliths from the vestibules without injury to the ampullæ the animal maintains its equilibrium in the water, rights itself promptly, and makes compensatory motions to rotations in all planes. If held in an abnormal position the compensatory position of the eyes is maintained. Both static and dynamic functions of equilibrium continue.

3. Destruction of both the semicircular canals and the otolith organs completely abolishes all compensatory movements and equilibrium reactions of labyrinthine origin.

4. It is pointed out that these observations do not justify the theory of Mach and Breuer that the ampullæ and semicircular canals are the organs for the dynamic functions of equilibrium, and that the otoliths are the organs for the static functions of equilibrium.

5. The new experiments recorded in this paper show that the ampullæ alone (without the otoliths) suffice for all the dynamic and all the static functions of equilibrium of the ear; and that the otolith organs (without the ampullæ) suffice for all the static and for all the dynamic functions of equilibrium of the ear with the exception of the response to a rotation of the animal in a horizontal plane.

STUDIES ON BIOLUMINESCENCE.

X. Carbon Dioxide Production during Luminescence of Cypridina Luciferin.

By E. NEWTON HARVEY.

(From the Physiological Laboratory, Princeton University, Princeton.)

(Received for publication, September 5, 1919.)

All luminous animals require oxygen for luminescence but it is not known whether they give off CO_2 as a result of the luminescence. Luminous bacteria produce carbon dioxide but this is no doubt the carbon dioxide of respiration. Even if we could prove that luminous bacteria produced more carbon dioxide than certain non-luminous bacteria, this would not necessarily mean that the excess carbon dioxide was produced during luminescence. Different organisms produce very different quantities of CO_2 per body weight and in no necessary relation to luminescence. In 1855, Fabre[1] published results which indicated that non-luminous portions of a mushroom, *Agaricus olearius*, produced less CO_2 (2.88 cc. of CO_2 per gm. in 36 hours at 12°C.) than luminous parts (4.41 cc. of CO_2 per gm. in 36 hours at 12°C.). There are, however, many reasons besides luminescence why one part of a plant might produce more CO_2 than another.

There are also many facts which indicate that luminescence is in no way connected with the respiration of cells. This is quite obvious in the case of animal cells where respiration is continuous and luminescence appears only as the result of stimulation of the cell. But even in bacteria where respiration and luminescence are both continuous, the two processes are distinct. Some luminous bacteria are facultative anaerobes and will multiply and respire but will not luminesce in the absence of oxygen.[2] McKenney[3] found that *Bacillus phosphorescens* will grow rapidly in 0.5 per cent ether without pro-

[1] Fabre, J. H., *Am. Sc. Nat.*, 1855, iv, 179.

[2] Beÿerinck, M. W., *Arch. Neer.*, 1889, xxiii, 416.

[3] McKenney, R. E. B., *Proc. Biol. Soc. Washington*, 1902, xv, 213.

ducing light. If the temperature of a luminous bacterium is gradually raised, respiration increases continuously up to a relatively high maximum, whereas the luminescence decreases rapidly above a relatively low optimum. The two processes are not equally affected by increasing temperature.

It is obvious that, to measure carbon dioxide production during luminescence, we must use cell-free solutions of the oxidizable material of luminous animals, luciferin, and oxidize it suddenly in order to have the maximum amount of CO_2 produced at one time. This experiment can be carried out with the luciferin of *Cypridina hilgendorfii*, an ostracod crustacean. A brilliant luminescence results from adding a small amount of luciferase solution to a solution of *Cypridina* luciferin. The preparation of these solutions has been described in a previous paper.[4]

Carbon dioxide production was tested by determining if any change in acidity, which might come from the CO_2 produced, occurs when solutions of luciferin and luciferase are mixed. After several attempts to measure acidity by adding an indicator (thymolsulfonephthalein) to the solution, this method was given up because the luciferin and luciferase solutions are yellowish in color, which interferes with the yellow-blue color change of the thymolsulfonephthalein. The electrometric determination with the hydrogen and 0.1 N KCl calomel electrode is the most sensitive. A McClendon electrode and Leeds and Northrup potentiometer were used. The acidity of the luciferin solution, luciferase solution, and the two after mixing was found to be the same, pH = 9.04. Therefore, not enough CO_2 is produced to affect the hydrogen ion concentration.

As both luciferin and luciferase solutions contain proteins and as luciferase is certainly and luciferin probably a protein, it will be seen that their buffer value is relatively high. The luciferin and luciferase solutions, although prepared with distilled water, no doubt contain also a small amount of buffer salts. Our experiments show this much, however, that not enough CO_2 is produced during luminescence to saturate the proteins in solution, including luciferin and luciferase themselves. The reaction responsible for luminescence, the oxida-

[4] Harvey, E. N., *J. Gen. Physiol.*, 1918–19, i, 269.

tion of luciferin, is, therefore, not to be compared to the reactions in cells giving rise to the carbon dioxide of respiration. As I have previously suggested[5] it is probably of a type similar to the oxidation of a leuco dye to the dye itself. In the case of methylene blue this reaction may be represented as follows:

$$\text{Leucomethylene blue } (C_{16}H_{20}N_3SCl) + O \leftrightarrows \text{methylene blue } (C_{16}H_{18}N_3SCl) + H_2O$$

In the case of luciferin we may have

$$\text{Luciferin } (LH_2) + O \leftrightarrows \text{oxyluciferin } (L) + H_2O$$

Whether the oxidation of luciferin really proceeds with the formation of H_2O or whether it involves the actual addition of oxygen, as in the oxidation of hemoglobin, is uncertain. Like methylene blue and oxyhemoglobin the oxyluciferin can be again reduced[5] but the reduction resembles that of methylene blue in that it will not take place by the mere removal of oxygen, as will the reduction of oxyhemoglobin, but requires also the addition of some reducing agent.

The fact that the reaction luciferin-oxyluciferin is reversible is in itself evidence that no carbon dioxide is produced. Reactions resulting in CO_2 production involve considerable energy changes (heat production) and are reversed only with the greatest difficulty and with the addition of a large amount of energy. Whether any considerable amount of heat is produced during oxidation of luciferin will be discussed in the following paper.

[5] Harvey, E. N., *J. Gen. Physiol.*, 1918–19, i, 133.

STUDIES ON BIOLUMINESCENCE.

XI. Heat Production during Luminescence of Cypridina Luciferin.

By E. NEWTON HARVEY.

(From the Nela Research Laboratory, National Lamp Works of the General Electric Company, Cleveland.)

(Received for publication, September 5, 1919.)

Dubois[1] obtained some evidence of infra-red radiation given off during the flash of the West Indian firefly, *Pyrophorus*. Langley and Very[2] also at first observed a slight heating of their bolometer from the light of the same insect, but in a later investigation, Langley[3] was unable to detect any heating of his bolometer whatsoever. Coblentz,[4] using a vacuum thermopyle, could detect no infra-red radiation from the American firefly, *Photinus*. Ives[5] also, using a phosphor-photographic method, found no infra-red radiation in the light of *Photinus*. We must attribute the earlier evidence to experimental errors and conclude that the light of luminous animals contains no rays of wave-length longer than the visible.

This does not mean, however, that no heat is produced by the reaction which produces the luminescence. A temperature change of a few thousandths or hundredths of a degree would evolve no measurable radiation. Coblentz[4] first studied the problem of heat production in the firefly, using a thermo-couple as the measuring instrument. He came to the conclusion that the temperature of the insect was slightly lower than the temperature of the air and that the luminous segments were slightly hotter than the non-lumi-

[1] Dubois, R., *Bull. Soc. Zool. France*, 1886, xi, 1.

[2] Langley, S. P., and Very, F. W., *Am. J. Sc.*, 1890, xl, series 3, 97.

[3] Langley, S. P., *Am. Astrophys. Obs.*, 1902, ii, 5.

[4] Coblentz, W. W., *Carnegie Institution of Washington, Publication No. 164*, 1912.

[5] Ives, H. E., *Phys. Rev.*, 1910, xxxi, 637.

nous segments. No definite increase or decrease in temperature could be established during the flash of the firefly. However, further work on the firefly is much to be desired.

The use of a living animal for such measurements introduces a possible source of error, in that any contraction of the muscles of the animal will produce heat which may add to an increase, or mask a decrease of temperature during luminescence. Utilization of extracts of luminous animals avoids the complications due to muscular contraction. From *Cypridina hilgendorfii*, a small crustacean, may be prepared a solution of luciferin, an oxidizable substance, and a solution of luciferase, a catalyzer which accelerates the oxidation of luciferin with light production. By bringing the solutions of luciferin and luciferase to the same temperature and then mixing them, one can measure any increase or decrease of temperature which occurs during the luminescence resulting from mixing. We can thus gain some idea of the heat of oxidation of luciferin.

Although the experiment sounds very simple it is actually somewhat difficult to carry out. The attainment of temperature equilibrium between two solutions is very slow when one wishes to obtain it to within 0.001°C. of the same temperature. After many attempts, the following arrangement of apparatus (Fig. 1) was found most satisfactory. About 10 cc. of luciferin solution were placed in the inner tube (D) of a special non-silvered thermos bottle (A). About 1 cc. of luciferase solution was placed in a very thin-walled glass tube (E) which was immersed in the luciferase solution and connected with a small motor so that it could be slowly but constantly rotated, thus stirring the solutions. Thermo-couples (L and M) of advance (0.008 inch) copper (No. 30, B and S enamel-insulated) wire were paraffined and placed in each tube and the copper wires connected through a copper double throw switch (C) with a Leeds and Northrup d'Arsonval wall galvanometer (No. 34,637, silver strip suspension) of 35 ohms resistance and 310 megohms sensitivity. The constant temperature junctions (N) were placed in a large Dewar flask (B) and filled with water at approximately the same temperature as the luciferin solution. 1 mm. galvanometer scale division represented 0.003°C. and the division readings could be estimated to tenths. By means of a glass rod (F) placed in the tube containing luciferase solution,

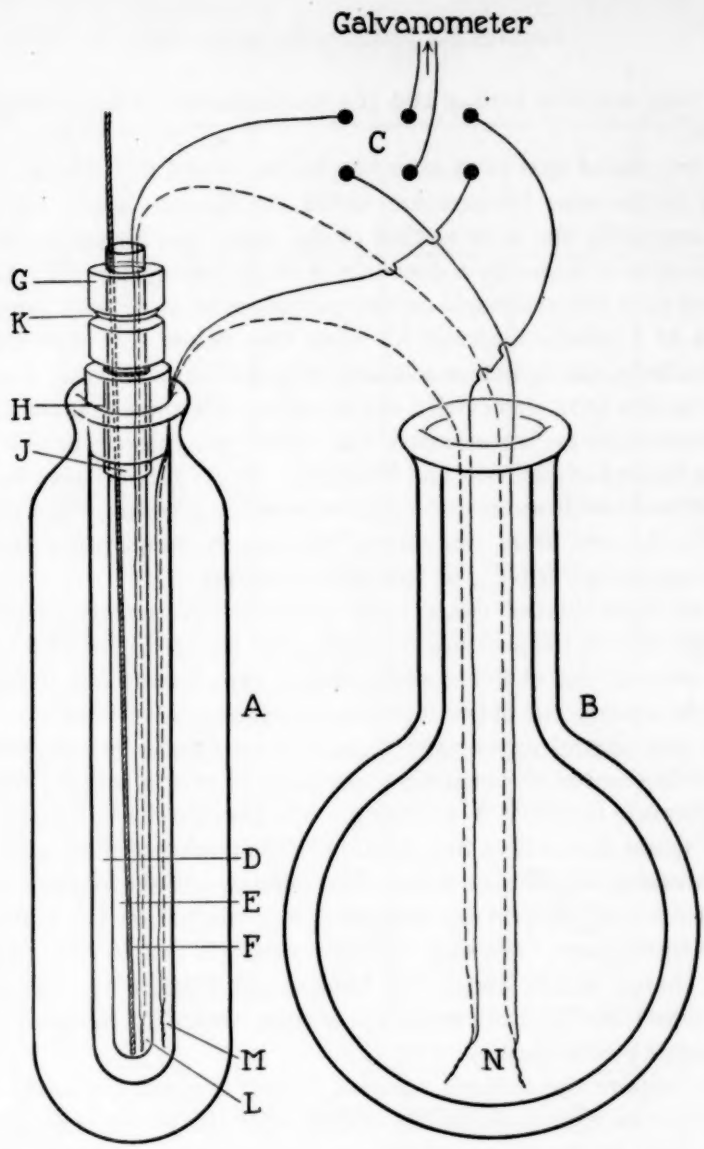

FIG. 1. Apparatus for determining heat production during luminescence of luciferin. A, special thermos tube. B, Dewar flask for constant temperature junctions. C, copper double throw switch. D, tube containing luciferin solution. E, tube containing luciferase solution. F, glass rod for breaking E. G, rubber stopper with groove, K, for pulley cord. H, cork closing thermos tube. J, brass sleeve in H allowing rotation of E. L, thermo-couple in luciferase solution. M, thermo-couple in luciferin solution. N, constant temperature junctions.

this tube could be broken and the luciferase and luciferin solutions mixed.

It was found that even after the luciferase and luciferin solutions came to the same temperature within the thermos bottle, this was not necessarily the same as that of the room, and a slow rise or fall occurred as indicated by a slow drift of the galvanometer coil. Readings of each thermo-couple on the galvanometer scale were therefore taken at 1 minute intervals for some time before and after mixing the luciferin and luciferase solutions and plotted as curves. Control experiments were also carried out in exactly the same manner as the luciferin-luciferase experiments but water was placed in the two tubes instead of luciferin and luciferase. Figs. 2 and 3 give typical experiments with water and with luminescent solutions respectively. As can be seen from the curves, the rise in temperature in each case figured is 0.006°C., or two scale divisions.

With both control (water) and luciferin experiments there was a slight rise in temperature on mixing the liquids in the two tubes. The average rise of five control (water) experiments was 0.0054°C. and the average rise of five luciferin experiments was 0.0048°C.

In one control experiment there was no change in temperature on mixing and in one luciferin experiment there was a slight drop in temperature (0.0045°C.) on mixing. The average rise in temperature is no doubt due to heat from friction in the mixing of the liquids and the breaking of the glass tube. The difference in the average rise of control and of luciferin experiments is so small (0.0006°C.) as to have little significance. We may therefore conclude that if any temperature change occurs during the luminescent reaction it is certainly less than 0.001°C. and probably less than 0.0005°C.; too small to be measured by this method.

To prepare the luciferin solution, 2 gm. of dried *Cypridina* were dissolved in 20 cc. of hot water and 10 cc. of this 10 per cent solution were used in the thermos bottle in the above experiments. If we assume that 1 per cent of the dried *Cypridina* is luciferin, 0.1 gm. of luciferin on oxidation was not able to change the temperature of the 10 cc. (in reality 11 cc., since 1 cc. of luciferase solution was mixed with the 10 cc. of luciferin) of solution 0.001°C. This means that 1 gm. of luciferin liberates less than 0.1 calorie during the luminescence accompanying oxidation.

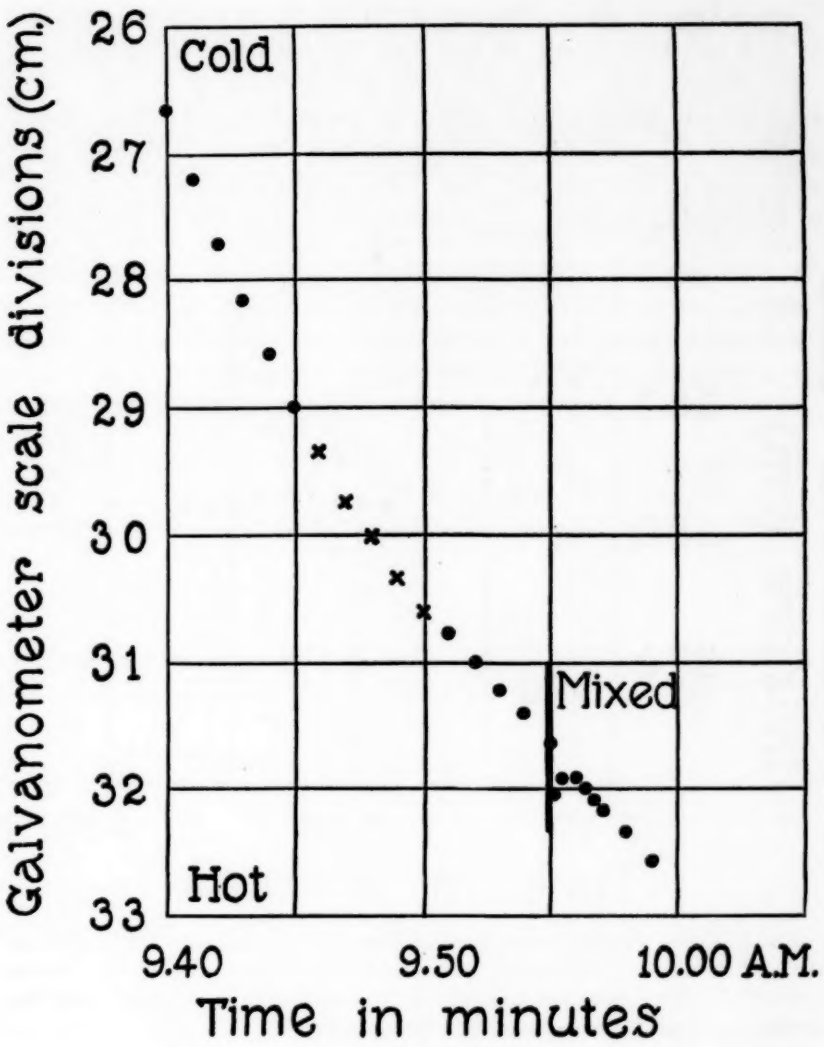

FIG. 2. Curve showing temperature change when two tubes containing water at the same temperature are mixed. 0.1 galvanometer scale division = 0.003°C. Dots represent readings of thermo-couple in tube D; crosses, readings of thermo-couple in tube E.

Since 1 gm. of glucose liberates 4,000 calories on complete oxidation to CO_2 and H_2O, it will be seen that the oxidation of luciferin is a very different type of reaction from the oxidation of glucose. As I have already suggested it is similar to the oxidation of reduced hemoglobin or the oxidation of leucomethylene blue to methylene blue. According to Barcroft and Hill[6] 1.85 calories are produced per gm. of hemoglobin oxidized. I have been unable to find figures for the

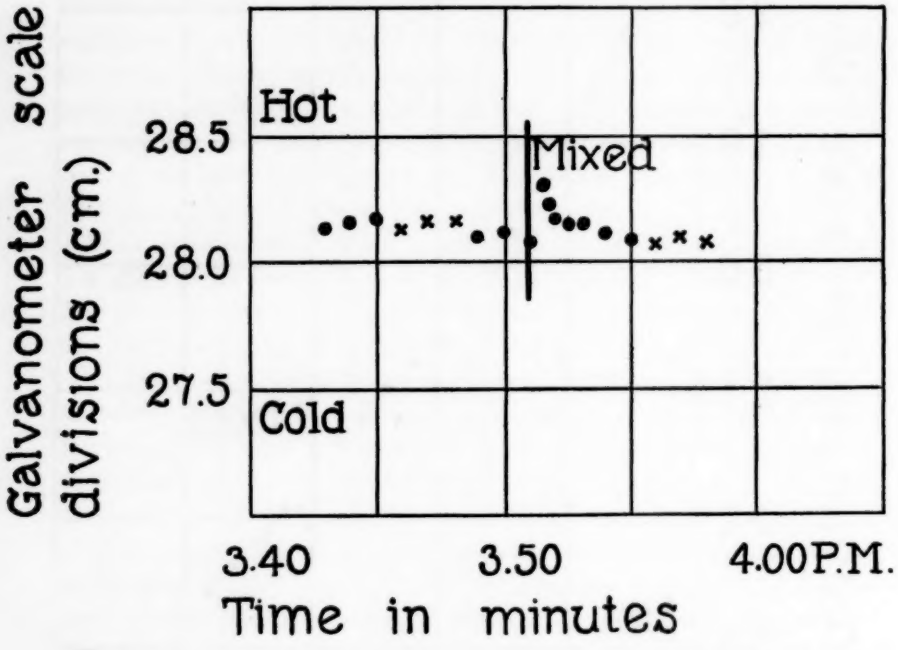

FIG. 3. Curve showing temperature change when luciferin and luciferase solutions at the same temperature are mixed. 0.1 galvanometer scale division =0.003°C. Dots represent readings of thermo-couple in luciferin solution; crosses, readings of thermo-couple in luciferase solution.

heat exchange during oxidation of leuco dyes, but it is no doubt also small.

The production of carbon dioxide always involves the evolution of considerable heat. Since luciferin evolves no measurable amount

[6] Barcroft, M. A., and Hill, A. V., *J. Physiol.*, 1909–10, xxxix, 411.

of heat on oxidation we have further evidence in support of that presented in a previous paper, that no carbon dioxide is produced during luminescence of luciferin. The energy change involved is very small indeed. It is on first thought surprising that so bright a luminescence as that of *Cypridina* should result from a reaction involving only a very small amount of heat. If we recall, however, that the eye is an extraordinarily sensitive instrument (responding to 10^{-9} ergs per second), which can detect light so weak, that, if it were converted into heat, would take 60,000,000 years to raise 1 gm. of water 1°C., we may realize that, after all, a very small heat production during oxidation of luciferin may be sufficiently great to account for its luminescence.

It gives me pleasure to acknowledge the kindly interest and assistance in setting up apparatus of Dr. W. Weniger of the Nela Research Laboratory.

THE ISOELECTRIC POINTS OF THE PROTEINS IN CERTAIN VEGETABLE JUICES.

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The preparation in the dry condition of systems rich in protein has assumed new importance in the practise of dehydrating vegetables and meats. Until recently the removal of water and the preservation of the original appearance of the foodstuffs have been considered the most important criteria for success in the operation. But acceptability of the dehydrated product has varied largely. In certain instances vegetables have lost important nutritional qualities upon dehydration or shortly thereafter. Others have been slow to reabsorb water. Occasionally dehydrated material has spoiled.

Information regarding the nature of the proteins in such systems should explain, in part, the causes of variation, set up criteria for change upon dehydration, and lead gradually to the perfection of processes.

With this in view the characteristics of the proteins in the juices of the potato, of the carrot, and of the tomato have been studied. Their isoelectric points and their solubility at different hydrogen ion concentrations have been determined. The acidity of the juices of these vegetables together with this information suggests the state in which the proteins exist in nature.

The Significance of the Isoelectric Point.

The classification of simple proteins depends upon their solubility in water containing different concentrations of inorganic salts (albumins from globulins), different concentrations of hydrogen ions (albumins and globulins from glutelins), or different concentrations of alcohol

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(prolamines).[1] But within each group state is affected by change in concentration of these factors. Thus the solubility of an albumin, a globulin, or a glutelin is variable with the amount of alcohol contained in the solvent; and the solubility of an albumin or a globulin is variable with the hydrogen ion concentration, and with the nature and amount of the dissolved electrolytes.

Recent investigations have suggested the course of certain of the chemical reactions that involve changes in the solubility of proteins. It appears that, whatever the superimposed complexity resulting from the colloidal nature of the systems, the behavior of protein substances, whether simple or conjugated, is dependent in large part upon their ionization as amphoteric electrolytes.[2,3,4] As amphoteric electrolytes, proteins combine with either acids or bases, but at a particular hydrogen ion concentration they exist most nearly uncombined. The value of this singular point in ionization and in behavior is characteristic of each protein and is termed its isoelectric point. The isoelectric point is thus a measure of the relative strength of protein as acid and base.

The combination of the protein molecule with acid or with basic radicles effects a change in its solubility and in its hydration. The compounds with simple acids or bases vary greatly in their ability to absorb water and to dissolve in water, but in the neighborhood of the isoelectric point protein substances are usually less soluble and less swollen than elsewhere. Empirical evidence for this conclusion antedates the theory for this phenomenon. Both in the laboratory and in industry the preparation of proteins has depended largely upon their lesser solubility near the isoelectric point.

The Determination of the Isoelectric Point.

The migration of charged particles in an electric field is termed cataphoresis. The charge of a protein depends upon its ionization.

[1] Recommendations of the Committee on Protein Nomenclature, *Am. J. Physiol.*, 1908, xxi, p. xxvii.

[2] Hardy, W. B., *Proc. Roy. Soc. London, Series B*, 1907, lxxix, 413. Sörensen, S. P. L., *Compt. rend. trav. Lab. Carlsberg*, 1917, xii.

[3] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 237.

[4] Henderson, L. J., Cohn, E. J., Cathcart, P. H., Wachman, J. D., and Fenn, W. O., *J. Gen. Physiol.*, 1918–19, i, 459.

Proteins can ionize as acids or as bases. As acids they migrate to the anode, as bases to the cathode. The nature of the ionization of the protein can thus be inferred from the direction of its migration. When it does not migrate in either direction the protein exists at its isoelectric point.

The isoelectric point of certain vegetable proteins was determined by the method of cataphoresis. The juice of the vegetable was placed in a U-tube, between electrodes charged with a potential difference, and the direction of the protein migration determined. The migration of protein during cataphoresis was followed both by determining nitrogen in the arms of the U-tube and by heating the liquid from the arms and noting in it the appearance of coagulated protein. The technique described by Coehn[5] and by Michaelis[6] was so modified as to meet the needs of the present research.

Unless several precautions are observed in investigating the direction and amount of the migration of protein compounds in an electric field the results are scarcely interpretable. The dipping of electrodes into the protein solution especially complicates the phenomenon[7] since proteins are either precipitated or rendered more soluble at the electrodes.[8] This is brought about, in part, by the accumulation of H^+ and OH^- ions at the cathode and anode respectively. Landsteiner and Pauli obviated these disturbances by using a three chambered vessel in determining the isoelectric point of very pure egg albumin.[9] Each chamber contained the protein solution, and migration was detected by determining nitrogen in each after the passage of current. Instead of protein solutions in the end-chambers Michaelis substituted a buffer solution of the same hydrogen ion concentration.

The particular form of apparatus devised for use in these experiments is represented in Fig. 1.[10] The protein solution was placed below and between the stop-cocks, BB, of a U-tube and above was

[5] Coehn, A., *Z. Electrochem.*, 1909, xv, 652.

[6] Michaelis, L., *Biochem. Z.*, 1909, xvi, 81.

[7] Haas, A. R. C., *J. Phys. Chem.*, 1918, xxii, 520.

[8] Robertson, T. B., *J. Phys. Chem.*, 1911, xv, 179.

[9] Landsteiner, K., and Pauli, W., *Verhandl. Kong. inn. Med.*, 1908, xxv, 571.

[10] The dimensions of the apparatus are of importance only in calculating movement of protein from the nitrogen concentration before and after cataphoresis. The volume of liquid contained in each arm was 7.4 cc. and in the central chamber below the arms (including stop-cocks) 13.4 cc.

placed a buffer solution of the same hydrogen ion concentration extending to stop-cocks, AA, and conveniently introduced at DD by creating a slight vacuum at C. Between the buffer solution (at A) and the zinc sulfate in the non-polarizable electrodes a sodium chloride solution was placed to avoid the formation of insoluble zinc salts. It was ascertained that the sodium chloride never met the protein by testing for the faster moving chlorine ion in a drop of solution pipetted from the arms of the U-tube. Once filled the level of the solutions was adjusted through C. The stop-cocks BB were then opened and, provided there was no diffusion, a 110 volt direct

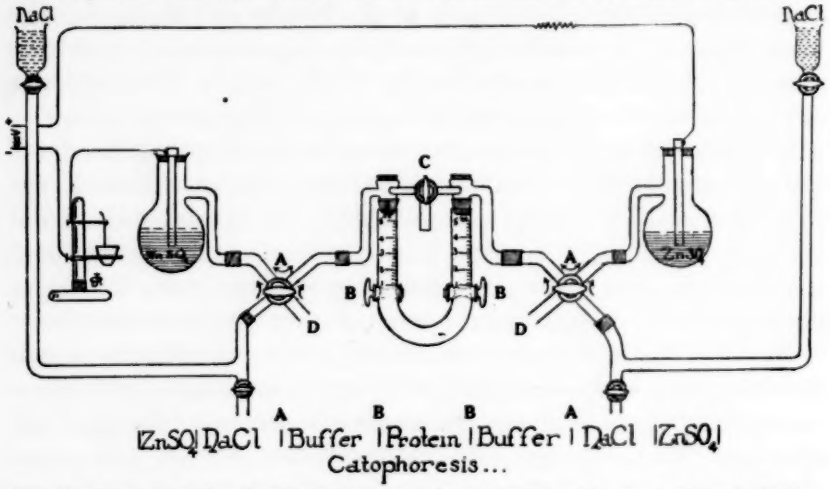

FIG. 1. Apparatus for the determination of protein migration in an electric field.

current was passed in series through the apparatus and a silver coulometer. The deposit of silver in the latter recorded the flow of current during cataphoresis. The drop in voltage across BB was approximately 3 volts, but varied in different experiments with the conductivity of the protein solution. Cataphoresis was carried on in a thermostat at 25° ± 1°C.

The Protein of the Potato.

About 8 per cent of the solids of the potato is protein and about 4 per cent is ash.[11] As a result, more than half the protein is dis-

[11] The average of all the values given in König, J., Chemie der menschlichen Nahrungs-und Genussmittel, Berlin, 4th edition, 1903, i, 713.

solved by the high concentration of electrolytes in the juice that can be squeezed from the potato. With the exception of a small amount of proteose only one well defined protein, the globulin tuberin,[12] has been isolated from the potato. It is present to the extent of from about 1 to 2 per cent in the juice, and was isolated and prepared from this and from the sodium chloride extract of the whole potato.

The Hydrogen Ion Concentration of Potato Juice.—The hydrogen ion concentration of the juice of the potato was slightly less than 10^{-6}N. When the juice was freed from suspended material—mostly starch—by filtration through pulp, the apparent hydrogen ion concentration was further reduced to about 10^{-7}N. It was then perfectly clear but darkened by the action of an oxidase intimately related to the globulin in location and in behavior. The oxidase was more active the lower the hydrogen ion concentration. A study of the action of the oxidase has been reported from this laboratory.[13]

The Precipitation of Tuberin —A precipitate separated upon the addition to the juice of the potato of either acid or alkali. The alkaline precipitate was less voluminous, more gelatinous, and more variable in amount than the acid precipitate. It was at first soluble in excess alkali, but later was denatured. Upon the addition of acid to potato juice a white, flocculent precipitate[14] appeared at about pH 6. The precipitate increased in amount and then gradually redissolved upon the addition of more acid. At acidities greater than those reported a protein precipitate gradually reappeared. This precipitate increased with increase in acidity and time and did not resemble a globulin in behavior.

The volumes of the precipitates that settled when different amounts of acid were added to potato juice in cylindrical vessels are recorded in Table I. In every instance the greatest precipitation appeared at a hydrogen ion concentration near 10^{-4}N. In bulk, however, and rate of settling, the precipitates varied to an extent which could not

[12] Osborne, T. B., and Campbell, G. F., *J. Am. Chem. Soc.*, 1896, xviii, 575.

[13] Falk, K. G., McGuire, G., and Blount, E., *J. Biol. Chem.*, 1919, xxxviii, 229.

[14] Upon standing, the protein frequently separated as a result of acid produced by bacteria.

be accounted for by differences in procedure. This variation was no greater than the variation in the amount of tuberin in the juice, but neither the amount of the precipitate nor its volume was dependent merely upon the concentration of protein. More probably it was related to differences in the content of electrolytes in the juice, for the addition of sodium chloride had a great effect upon the behavior both of the acid and of the alkaline precipitate.

TABLE I.

Measurements on Potato Juice.

N HCl added to 100 cc. of potato juice.	Gm. of nitrogen in potato juice.															
	0.213	0.238	0.242	0.292	0.309	0.310	0.213	0.238	0.286	0.315	0.213	0.238	0.242	0.309	0.310
	Volume of protein precipitate.						Nitrogen in filtrate from precipitate.				pH of filtrate from precipitate.				
cc.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*gm.*	*gm.*	*gm.*	*gm.*					
16		0						0.225							
15				0	0	1			0.275					2.18	2.30
13	9						0.210				2.90				
12						7									2.74
10	25			7	17		0.178		0.242		3.15			2.95	
8		12	80	22	54	20		0.182	0.243			2.75	3.45	3.35	3.57
7	84						0.179				3.60				
6			93	61	64				0.239	0.240			3.85	3.84	
5	88		96		68	23	0.187		0.251	0.242	4.27		4.27	4.12	4.32
4		30	91	30	82			0.200	0.251	0.248		4.07		4.39	
3	92				56	17	0.204		0.251	0.249	4.80			4.73	4.93
2		18			18			0.199		0.255		4.60		5.35	
1		4			0	0		0.220				5.45		6.31	6.15
0	0	0		0	0	0	0.213	0.238	0.286	0.315		6.38		7.00	6.80
N NaOH															
1		4			12	6				0.291		7.65		7.78	7.55
2		9			15	6				0.290		8.66		8.22	8.19
4		5			16	6				0.293		9.50		8.84	8.89
6		3			18	7				0.301				9.39	9.35
8					34									9.78	

This is illustrated by the experiment recorded in Table II in which different amounts of sodium chloride were added to juice to which the same amount of hydrochloric acid had been added. The rates of settling and the volumes of the resulting precipitates were observed

and the nitrogen content of the juices was determined after the precipitate had been removed by centrifuging. Small amounts of sodium chloride greatly decreased the volume of the precipitate and also increased the solubility of the tuberin, though not to the same extent. The effect of sodium chloride upon the solubility of tuberin and upon the apparent hydrogen potential of systems containing it is different not only in amount but in kind at different hydrogen ion concentrations. The relation between this change and the isoelectric point will be considered in another report.

TABLE II.

Effect of Sodium Chloride upon the Precipitation of Tuberin by Acid.

NaCl added to 100 cc. of potato juice + 5 cc. of N HCl.	Volume of precipitate.	Nitrogen in filtrate.
gm.	*cc.*	*gm.*
0	97	0.192
0.5	98	0.195
1.0	21	0.204
2.0	20	0.199
4.0	29	0.196
8.0	61	0.178

The Solubility of Tuberin.—The solubility of tuberin at different hydrogen ion concentrations was estimated by determining the nitrogen content of potato juice to which sodium hydroxide or hydrochloric acid had been added in different amounts and from which any resulting precipitate had been removed by filtering or centrifuging. The results of four different experiments, in two of which the volumes of the precipitate were also determined, are reported in Table I. In each tuberin was least soluble in the juice of the potato at hydrogen ion concentrations slightly greater than 10^{-4}N, where precipitation occurred. Precipitation also occurred at hydrogen ion concentrations near 10^{-8}N. The amount of these precipitates was very different, but neither the addition of acid nor of alkali completely precipitated the protein in potato juice. Indeed no more than 25 per cent of the total nitrogen in the juice of the potato was ever found in the most copious acid precipitate nor more than 8 per cent in the largest alkaline precipitate. A curve representing the solubility of tuberin in potato juice thus passes through two minima and a maximum, the latter at the reaction of potato juice (Fig. 2).

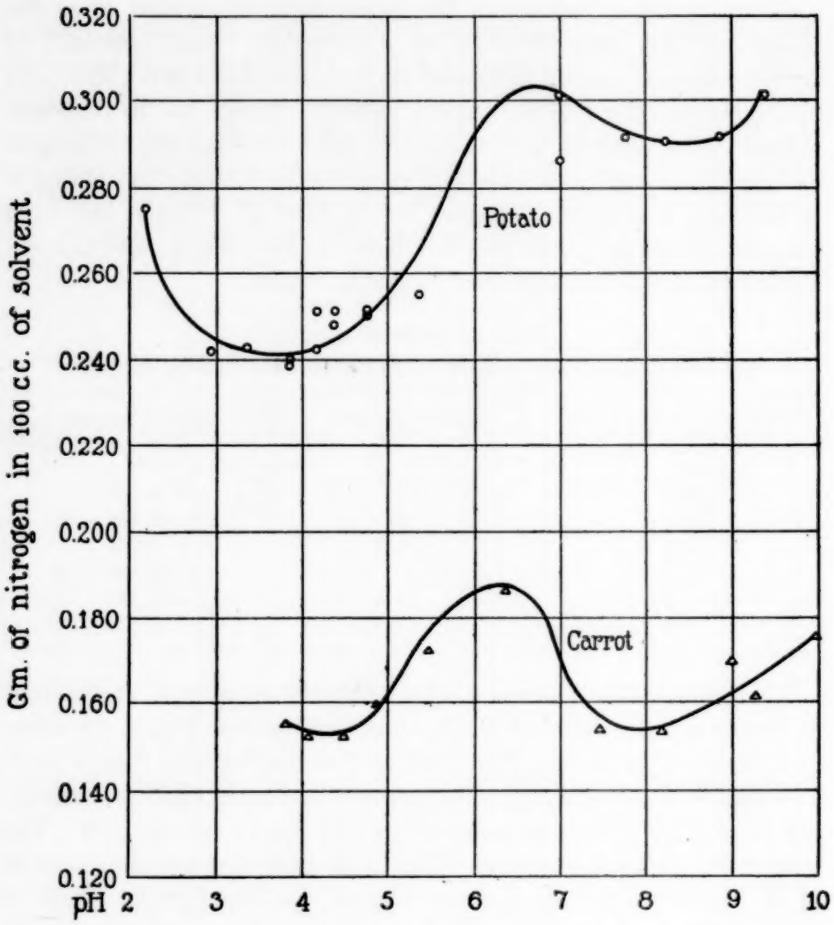

FIG. 2. Solubility of protein in vegetable juices at different hydrogen ion concentrations.

The Compounds of Tuberin.—The changes in the solubility of tuberin that have been described resulted, in part, from the combination with acids and bases of the basic and acid radicles in the juice of the potato. The amount of such combination is calculable if the hydrogen ion concentration is determined. It is equal to the difference between the measured hydrogen ion concentration and

that which would have resulted from the added acid or alkali had there been no combination. It is indicated by the changing slope of the curve representing the titration if the amount of normal acid or alkali is represented as ordinate and the hydrogen ion concentration as abscissæ (Fig. 3).

The curve representing the titration of potato juice with acid and alkali passes through a point of inflection at a hydrogen ion

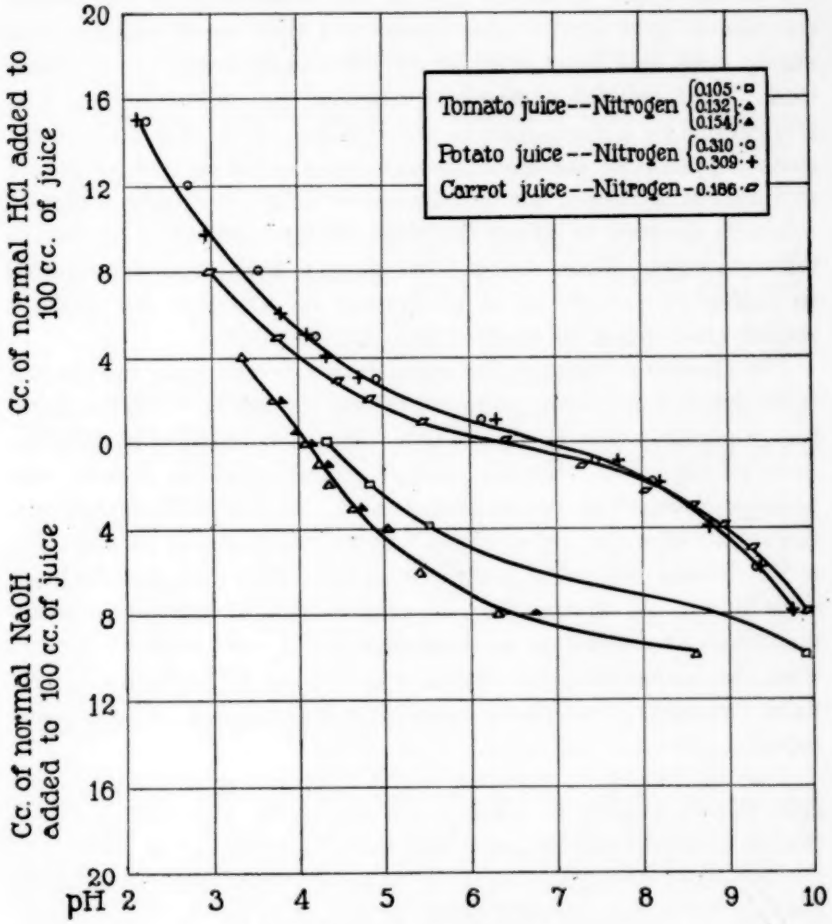

FIG. 3. Titration of vegetable juices.

concentration near the second ionization constant of phosphoric acid (1.6×10^{-7} = pH 6.8). But the strong buffer action, indicated by the steepness of the titration curve, throughout the range investigated cannot be attributed merely to the presence of phosphoric acid nor to the other organic and inorganic weak acids that chemical analyses reveal. The increase in steepness of the titration curve in the range, acid to pH 4.5 on the one hand and alkaline to pH 8.5 on the other, is largely due to the dissociation of the protein compounds that exist in the potato and their recombination with strong acids and bases with the retention of hydrogen and hydroxyl ions. The formation of new compounds at these particular reactions is suggested by a comparison of the titration curve (Fig. 3) with the curve representing the solubility of tuberin in the juice of the potato at different hydrogen ion concentrations (Fig. 2). Each change in solubility is seen to affect the slope of the titration curve, but in different ways. Thus, nearly three times as much acid was required to redissolve tuberin as to precipitate it. Osborne has made a similar observation on another globulin, edestin.[15]

The Isoelectric Point of Tuberin.—In an electric field the protein in the juice of the potato migrated toward the anode. It bore, therefore, a negative charge. No change followed an increase in the alkalinity of the juice. The direction of migration of the protein was, however, reversed by the addition of acid. In Table III are collected the results of many experiments. In all the direction of migration of the protein changed at a slightly lower hydrogen ion concentration than 10^{-4}N. At that acidity the protein did not migrate in either direction. It existed at its isoelectric point. At acidities greater than the isoelectric point protein migrated to the cathode. Under these circumstances tuberin apparently ionized as a base, and dissolved as an acid compound.

A second change in direction of the migration of protein in very acid juice occasionally occurred in our early experiments. This change was probably apparent and must be explained as relative to the increased passage of water at these acidities, since it was later averted by increasing the buffer in the arms of the cataphoresis apparatus.

[15] Osborne, T. B., *J. Am. Chem. Soc.*, 1902, xxiv, 39.

The flow of current during cataphoresis was measured, as has been said, by a silver coulometer in series with the apparatus. Since the juice of the potato contains both free electrolytes and electrolytes combined with tuberin, deductions regarding the transference of current by tuberin cannot readily be drawn from these data (Table III). It would be reasonable to suppose, however, that as the acidity of the solutions increased their specific conductivity would be increased

TABLE III.

Migration of Protein in Potato Juice during Cataphoresis.

N HCl added to 100 cc. of potato juice.	Amount of nitrogen in 5 cc. of filtrate.	pH of filtrate.	Conditions of cataphoresis.			Protein migration during cataphoresis.			
			Buffer solution used (M/20).		Flow of current.	Direction.	Amount of nitrogen after cataphoresis in 5 cc.		
			$C_2H_4O_2$	$C_2H_3O_2Na$	Silver per hr.		Anodal chamber.	Central chamber.	Cathodal chamber.
cc.	*mg.*		*cc.*	*cc.*	*gm.*		*mg.*	*mg.*	*mg.*
16			*			Cathodic.			
10	9.4	3.10	19.6	0.4	0.0091	"	(0.2	4.7	5.2)‡
8		3.45	19.0	1.0	0.0060	"			
7	8.4	3.60	18.5	1.5	0.0145	"	0.7	8.0	0.8
6		3.85	17.0	3.0	0.0185	"			
5	8.9	4.27	14.0	6.0	0.0338	"	0.5	7.2	0.6
5	11.1	4.27	14.0	6.0	0.0253	"			
4	12.1	4.50	12.0	8.0	0.0050	Anodic.	3.9	9.9	0.3
3		4.80	8.0	12.0	0.0048	"			
0	14.6	6.22	2.0†	8.0		"			

* Citrate mixture used.

† M/60 phosphate mixture used.

‡ Cataphoresis continued for 17½ hours.

because of the high mobility of hydrogen ions. None the less, the flow of current during cataphoresis seems to pass through a maximum at a hydrogen ion concentration slightly less than 10^{-4}N (Table III). Presumably conductivity passes through a maximum in the neighborhood of the isoelectric point.

The hydrogen ion concentration which numerically represents the isoelectric point of tuberin reveals the relatively stronger acid proper-

ties of this protein. The amount of acid required to titrate the protein to its isoelectric point is a measure of the amount of combined protein that exists in the juice of the potato and is dissociated by the addition of acid. The soluble salt of this protein that is formed on the other side of its isoelectric point evidently contains three times as much acid as was necessary to dissociate the compound that existed in nature. The relation between this compound and those that exist at other hydrogen ion concentrations and the state in which they exist are suggested by these data.

The Protein of the Carrot.

Remarkably similar in behavior to potato juice is the juice of the carrot. Existing at approximately the same hydrogen ion concentration it combines acid and alkali to precisely the same extent. Fig. 3 illustrates the essential coincidence of their titration curves. Moreover, protein separates from the juice of the carrot upon the addition either of acid or of alkali. The volume of the precipitate and the nitrogen in the filtrate are both recorded in Table IV. As in the potato, the acid precipitate of greatest bulk appeared at a hydrogen ion concentration slightly less than 10^{-4}N. The alkaline precipitate appeared near 10^{-8}N.

The isoelectric point of the protein in juice filtered from the acid precipitate was determined. The change in direction of protein migration also occurred at a hydrogen ion concentration not far from 10^{-4}N. All the measurements upon carrot juice are arranged in tabular form. With one exception—derived from the data upon its solubility—the salient characteristics of carrot protein suggest those of tuberin. The isoelectric point and amphoteric constants of the protein in both vegetables are essentially identical. In nature they exist at approximately the same reaction. A consideration of the curves representing their solubility at different hydrogen ion concentrations suggests that they may exist as somewhat similar compounds (Fig. 2). But whereas in the potato the alkaline precipitate is slight, in the carrot it is almost as great in amount as the acid precipitate.

TABLE IV.

Measurements on Carrot Juice.

N HCl added to 100 cc. of carrot juice.	Gm. of nitrogen in carrot juice.						Direction of protein migration.
	0.186	0.261	0.186	0.186	0.261	0.276	
	Volume of protein precipitate.		Nitrogen in filtrate from precipitate.	pH of filtrate from precipitate.			
cc.	*cc.*	*cc.*	*gm.*				
20		27			1.65		
16		26			2.21	2.10	Cathodic.
12		28			3.07	2.57	
10							
8	13	30		2.98		3.22	Cathodic.
6							
5	15		0.155	3.80			
4	17	36	0.152	4.06	4.37	4.23	
3	19		0.152	4.48	4.72		Anodic.
2	8		0.159	4.86			
1	0		0.172	5.45			Anodic.
0	0	8	0.186	6.43		6.25	"
N NaOH							
1	2		0.153	7.45			
2	5		0.153	8.18			
3	6		0.159	8.67			
4	6		0.169	8.95			
5	8		0.161	9.26			
8	14		0.175	9.92			

The Protein of the Tomato.

The juice of the tomato is very different. A high concentration of organic acids give to the ripe tomato a hydrogen ion concentration of nearly 10^{-4}N. According to Albahary,[16] the principal acids are malic, phosphoric, and citric. According also to Albahary,[17] the concentration of "albumin" and of "nucleoprotein" decreases during the period of maturation of the tomato and the concentration of free acids increases. As a result of a study of the buffer process in the metabolism of plants, Miss Hempel also suggested that the

[16] Albahary, J.-M., *Compt. rend. Acad. Sc.*, 1908, cxlvi, 336.

[17] Albahary, F.-M., *Compt. rend. Acad. Sc.*, 1908, cxlvii, 146.

extremely high acidity of the ripened lemon might be brought about in some such way.[18]

The lack of homogeneity of tomato juice that has merely been squeezed through cheese cloth is manifest. It contains, however, nearly 1 per cent of protein. Filtering the juice removes a large amount of this protein and at the same time appears to decrease the hydrogen ion concentration of the filtrate to nearly 10^{-5}N. Whatever

TABLE V.

Measurements on Tomato Juice.

N HCl added to 100 cc. of tomato juice.	Gm. of nitrogen in tomato juice.								
	0.105	0.132	0.154	0.105	0.132	0.154	0.105	0.132	0.154
	pH of tomato juice.			Direction of protein migration.			Silver deposited during cataphoresis.		
cc.							gm.	gm.	gm.
4		3.38							
2		3.71	3.80					0.0100	0.0380
1			4.00			Cathodic.			0.0380
0.6						"			0.0160
0	4.35	4.13	4.15	Cathodic.	Cathodic.		0.0609	0.0481	0.0243
N NaOH									
1		4.24	4.32			Cathodic.			0.0481
2	4.85	4.39		Cathodic.			0.0467		
3		4.63	4.79			Anodic.			0.0204
4	5.54	5.05		Anodic.	Anodic.		0.0321	0.0260	
6		5.42							
8		6.35	6.76						
10	9.90	8.68							
20	11.95								

the explanation of the latter observation, the removal of protein by filtration is easily understood. Protein exists largely in suspension.

In entire conformity are the results of cataphoresis (Table V). The unfiltered juice usually migrates to the cathode. A slight reduction in acidity reverses the sign of the protein. The protein, therefore, exists near its isoelectric point and probably slightly on the acid

[18] Hempel, J., *Compt. rend. trav. Lab. Carlsberg*, 1917, xiii.

side of it. In more nearly neutral solution it is soluble and can be freed from other constituents of the tomato by filtering through pulp. It then migrates to the anode. The addition of acid again precipitates the protein at its isoelectric point.

SUMMARY.

The state in which a protein substance exists depends upon the nature of its combination with acids or bases and is changed by change in the protein compound.

The nature of the compound of a protein that exists at any hydrogen ion concentration can be ascertained if the isoelectric point of the protein is known.

Accordingly information regarding the isoelectric points of vegetable proteins is of importance for operations in which it may be desirable to change the state of protein substances, as in the dehydration of vegetables.

The Protein in Potato Juice.—The hydrogen ion concentration of the filtered juice of the potato is in the neighborhood of 10^{-7}N. Such juice contains the globulin tuberin to the extent of from 1 to 2 per cent.

The character of the compound of tuberin that exists in nature was suggested by its anodic migration in an electric field.

The addition of acid to potato juice dissociated this compound and liberated tuberin at its isoelectric point. The isoelectric point of tuberin coincided with a slightly lower hydrogen ion concentration than 10^{-4}N. At that reaction it existed most nearly uncombined.

The flow of current during cataphoresis was greatest in the neighborhood of the isoelectric point. This evidence supplements that of the direction of the migration of tuberin, since it also suggests the existence of the greatest number of uncombined ions near this point.

At acidities greater than the isoelectric point tuberin combined with acid. The compound that was formed contained nearly three times as much acid as was needed to dissociate the tuberin compound that existed in nature. At such acidities tuberin migrated to the cathode.

Though never completely precipitated tuberin was least soluble in the juice of the potato in the neighborhood of its isoelectric point.

Both the compounds of tuberin with acids and with bases were more soluble in the juice than was uncombined tuberin.

The nature of the slight precipitate that separated when potato juice was made slightly alkaline was not determined.

The Protein in Carrot Juice.—The isoelectric point of the protein in carrot juice coincided with that of tuberin. Remarkably similar also were the properties of carrot juice and the juice of the potato. Existing in nature at nearly the same reaction they combined with acids and bases to nearly the same extent and showed minima in solubility at the same hydrogen ion concentrations. The greatest difference in behavior concerned the alkaline precipitate which, in the carrot, was nearly as great as the acid precipitate.

The Protein in Tomato Juice.—The protein of the tomato existed in a precipitated form near its isoelectric point. Accordingly it was not present to any extent in filtered tomato juice. If, however, the considerable acidity at which the tomato exists was neutralized the protein dissolved and was filterable. It then migrated to the anode in an electric field. The addition of sufficient acid to make the hydrogen ion concentration slightly greater than 10^{-5}N again precipitated the protein at its isoelectric point. At greater acidities migration was cathodic.

IODINE AND THE THYROID.

IV. Quantitative Experiments on Iodine Feeding and Metamorphosis.

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(Received for publication, September 15, 1919.)

This paper deals with experiments undertaken with the object of determining, by quantitative feeding, the approximate amount of elemental iodine necessary to induce complete metamorphosis in normal and thyroidless toad larvæ, reared under identical environmental conditions.

In previous studies on the relation of iodine to the thyroid,[1] as determined by feeding this halogen and its compounds to tadpoles, the experimental evidence led to the suggestion that probably the chief function of the thyroid apparatus, at any rate in the Anura, is the extraction from the blood, and storage of the extremely minute quantities of iodine taken into the body by means of food and water, and the subsequent release of this substance perhaps in a modified form, as the thyroid hormone. The purpose of this experiment was to test the validity of this conclusion, for it is evident, that if the thyroid apparatus in Anura serves the purpose of collecting, storing, and transforming the incoming iodine into a more active hormonic agent, then tadpoles possessing normal thyroid glands should react more promptly by metamorphosis to iodine feeding than tadpoles of similar age and parentage, whose thyroids had been removed before the period of their first functioning, and hence were devoid of the requisite mechanism for collecting, storing, and transforming iodine. Moreover, it was hoped that such an experiment would show whether or not there is an irreducible minimum quantity of iodine required by anurans for metamorphosis.

[1] Swingle, W. W., *J. Exp. Zool.*, 1918–19, xxvii, 397, 417; *J. Gen. Physiol.*, 1918–19, i, 593; *Endocrinology*, 1918, ii, 283.

If this proved to be true, then the minimum figure should be much lower for animals with thyroid glands than for those without.

A brief resumé of the facts brought to light in earlier papers on this subject are: (1) Inorganic iodine and its compounds, iodoform and potassium iodide, greatly accelerate metamorphosis of tadpoles. (2) Anuran larvæ from which the thyroid gland had been removed at its inception (*i.e.* 6 mm. larvæ) and which under normal conditions do not undergo metamorphosis, but grow to abnormal size, quickly transform into frogs when fed elemental iodine. This indicated that iodine is essential for amphibian metamorphosis, that it is the active constituent of the thyroid glands of these animals, and, judging by its physiological effect on thyroidless tadpoles, that it exerts its activity directly upon the cells and tissues of the organisms; *i.e.*, that iodine is capable of functioning like the thyroid hormone in thyroidless tadpoles, and the thyroid-like action is either inherent in the iodine atom, or else the elemental iodine is transformed into a substance similar to thyroid secretion by tissues other than those which normally perform this function. (3) The thyroid follicles of tadpoles on an iodine diet show a greater colloid content than do the glands of normally fed animals. (4) The peculiar action of iodine in accelerating metamorphosis is apparently unique and not possessed by other closely related chemical agents like bromine. (5) Anuran metamorphosis depends upon the amount of iodine secured by the larvæ; the greater the quantity the more rapid the differentiation.

Materials and Observations.

The experimental animals were tadpoles of the common toad, *Bufo lentiginosus;* all came from the same egg mass and hence presumably were of the same parentage. The thyroid anlagen of 100 animals were extirpated at the time of their evagination from the pharyngeal floor, and the larvæ appropriately controlled.[2] When the larvæ were 9 mm. long, control and thyroidless groups were divided into five cultures each, twenty larvæ to a culture. The containers were large, glass bowls containing 3,000 cc. of tap water. Four thyroidless cultures and four cultures of normal animals were used for experimental purposes; the remaining two cultures served as

[2] The writer is indebted to Miss M. E. Larson for the larvæ.

checks. The original plan was to use only distilled water, but water distilled by ordinary methods was found toxic for tadpoles. Only quartz distilling was satisfactory. Unfortunately the laboratory facilities for this method were inadequate for producing 30,000 cc. daily, so tap water was substituted. Princeton water possibly contains minute traces of iodine, but if so it is present in such small quantities as to prove negligible in the present experiment.

The nature and amount of the food fed were carefully controlled, and consisted of fat-free casein, yeast, potato starch, and rolled oats. 3 days a week small quantities of *Elodea* were placed in the containers. Toad larvæ do not feed upon the *Elodea* itself, but only upon the diatoms and other organisms living on it. It is evident, from the choice of foods listed, that the possibility that iodine might be present in minute traces was not absolutely ruled out. However, as in the case of the tap water, if present it was insufficient in quantity to affect metamorphosis during the interval the experiment lasted. The water of the cultures was changed daily and the containers were well rinsed. An M/20 stock solution of iodine was employed. The gram concentration of iodine used for the various cultures is given in the following table. This amount was given daily.

Thyroidless cultures.	Concentration of iodine.	Normal cultures.	Concentration of iodine.
Culture 1.....	0.0000014532	Culture 1.....	0.0000014532
" 2.....	0.0000011499	" 2.....	0.0000011499
" 3.....	0.0000007666	" 3.....	0.0000007666
" 4.....	0.0000003833	" 4.....	0.0000003833

Frog and toad larvæ are said not to swallow water[3] but to absorb it through the integument, consequently this method of iodine administration is very satisfactory. The obvious defect of this method is that it is impossible to determine the exact amount of water absorbed or iodine obtained by the larvæ per day.

For the sake of brevity, all descriptions of the various cultures will be presented in tabular form. Date of first iodine administration, May 19; average total length 9 mm.; no limb buds present. A plus sign indicates that fore limbs have appeared.

[3] Boulenger, E. G., Reptiles and batrachians, London and New York, 1914, xiv, 197–199.

Observations of May 26.

Thyroidless iodine-fed.	Total length.	Hind legs.	Fore legs.	Tail atrophy.	Condition.	Mortality.
	mm.	mm.				
Culture 1	15.0	0.80		None.	Good.	None.
" 2	16.0	0.65		"	"	"
" 3	17.0	0.43		"	"	"
" 4	16.5	0.36		"	"	"
Normal iodine-fed.						
Culture 1	14.5	2.0 (Toes.)		None.	Thin.	None.
" 2	15.5	2.0 (No toes.)		"	"	"
" 3	17.0	1.0 " "		"	Good.	"
" 4	16.0	0.84		"	"	"
Thyroidless control	15.0	0.30		"	"	"
Normal "	14.0	0.46		"	"	"

Observations of June 4.

Thyroidless iodine-fed.	Total length.	Hind legs.	Fore legs.	Tail atrophy.	Condition.	Mortality.
	mm.	mm.				
Culture 1	19.5	3.5			Emaciated.	2
" 2	20.5	2.5			"	
" 3	22.0	1.5			Good.	1
" 4	21.0	1.0			"	
Normal iodine-fed.						
Culture 1	18.5	5.0			Emaciated.	
" 2	19.0	4.0			"	1
" 3	21.5	3.0			"	
" 4	20.0	1.5			Good.	
Thyroidless control	18.5	0.94			"	
Normal "	17.5	0.98			"	1

Observations of June 18.

Thyroidless iodine-fed.	Total length.	Hind legs.	Fore legs.	Tail atrophy.	Condition.	Mortality.
	mm.	mm.				
Culture 1	24.5	8.5			Emaciated.	
" 2	28.0	8.0			"	
" 3	29.0	4.0			Good.	1
" 4	27.0	2.5			"	
Normal iodine-fed.						
Culture 1	Completed metamorphosis, June 12 to 18.					
" 2	21.5	11.0	*	+	Emaciated.	2
" 3	23.0	7.5			"	
" 4	22.5	5.0			Good.	
Thyroidless control	25.0	1.5			"	
Normal "	21.5	1.5			"	

* Fore limbs of three animals breaking through the skin; culture metamorphosing.

Observations of June 25.

Thyroidless iodine-fed.	Total length.	Hind legs.	Fore legs.	Tail atrophy.	Condition.	Mortality.
	mm.	*mm.*				
Culture 1	27.5	10.0	Through.*	+	Thin.	2
" 2	30.5	9.5	None.		"	1
" 3	32.0	5.5	"		Good.	
" 4	29.5	3.0	"		"	
Normal iodine-fed.						
Culture 1	Completed metamorphosis, June 12 to 18.					
" 2	"		"	"	18 " 23.	
" 3	24.0	10.5			Thin.	3
" 4	24.5	7.0			"	
Thyroidless control	28.0	2.0				1
Normal "	22.5	3.0				1

* Fore limbs through skin in six animals; culture completed metamorphosis by une 29. Four animals died before resorption of the tail was complete.

It will be noted that Cultures 1 and 2 of the normal iodine-fed larvæ, living in iodine concentrations of 0.0000014532 and 0.0000011499 respectively, completed metamorphosis before the corresponding thyroidless cultures began to transform. It will be shown later that the interval between metamorphosis of normal iodine-fed and thyroidless iodine-fed cultures in equivalent solutions is lengthened the lower the iodine concentration, finally becoming perhaps an indefinite interval in the case of the thyroidless animals.[4] This strongly indicates that there is an irreducible minimum of iodine concentration for metamorphosis, beyond which thyroidless larvæ are unable to utilize the minute quantities of iodine present, though this may be sufficient to stimulate metamorphosis in animals with intact thyroid apparatus.

[4] Thyroidless Cultures 3 and 4 unfortunately were lost September 11, owing to neglect to change the water for 52 hours, resulting in decay of the food, especially casein, and consequent death of the larvæ. At this time none of the animals in Culture 4 showed indications of metamorphosis. However, two animals of Culture 3 were about to transform.

Observations of July 5.

Thyroidless iodine-fed.	Total length.	Hind legs.	Fore legs.	Tail atrophy.	Condition.	Mortality.
	mm.	*mm.*				
Culture 1	Completed metamorphosis, June 25 to 29.					
" 2	33.0	11.5	Through.*	+	Emaciated.	1
" 3	34.5	6.0			"	1
" 4	33.0	4.5				
Normal iodine-fed.						
Culture 1	Completed metamorphosis, June 12 to 18.					
" 2	"		"		" 18 " 23.	
" 3	"		"		" 28 " July 5.	
" 4	26.5	8.0			Thin.	2
Thyroidless control	33.0	3.0				1
Normal "	25.0	3.5				

* Fore limbs through skin in three animals of thyroidless Culture 2; culture began metamorphosing July 3 and completed the transformation by July 10.

Observations of July 19.

Thyroidless iodine-fed.	Total length.	Hind legs.	Fore legs.	Tail atrophy.	Condition.	Mortality.
	mm.	*mm.*				
Culture 1	Completed metamorphosis, June 25 to 29.					
" 2	"		"		July 3 " 10.	
" 3	38.0	6.5			Good.	
" 4	37.0	5.0			"	
Normal iodine-fed.						
Culture 1	Completed metamorphosis, June 12 to 18.					
" 2	"		"		" 18 " 23.	
" 3	"		"		" 28 " July 5.	
" 4	28.0	10.0			Thin.	
Thyroidless control	36.0	4.5			Good.	
Normal "	26.0	4.0			"	

Observations of July 30.

Thyroidless iodine-fed.	Total length.	Hind legs.	Fore legs.	Tail atrophy.	Condition.	Mortality.
	mm.	*mm.*				
Culture 1	Completed metamorphosis, June 25 to 29.					
" 2	"		"		July 5 " 10.	
" 3	43.0	7.0			Good.	2
" 4	40.0	6.5			"	1
Normal iodine-fed.						
Culture 1	Completed metamorphosis, June 11 to 18.					
" 2	"		"		" 18 " 23.	
" 3	"		"		" 28 " July 6.	
" 4	"		"		July 27 " August 2.	
Thyroidless control	42.5	5.0			Good.	1
Normal "	28.5	5.5			"	1

By July 30, all the normal iodine-fed animals in Cultures 1, 2, and 3 had completed metamorphosis, while in Culture 4 many of the larvæ were transforming, and all had completed the process by August 2. Of the thyroidless animals, Cultures 1 and 2 had metamorphosed earlier, but Cultures 3 and 4 showed on this date no very marked indications of assuming adult characters very soon. These two cultures were fed and tended as usual until September 5. During this interval the animals increased considerably in size, those in Culture 3 averaging 52 mm. with hind legs 10 mm. long; the larvæ of Culture 4 averaged 47 mm. with hind legs 7.5 mm. There were no marked indications of metamorphosis. The two control cultures, one normal and the other thyroidless, averaged in length 27.5 mm. and 40 mm. respectively, with hind legs 7.5 mm. and 5.5 mm. Growth in these two cultures was very slow after July and practically ceased by August 2. The diet appears responsible for this, in part at least. In earlier papers[1] the writer has shown that when fed in minimum quantities iodine stimulates growth in anuran larvæ. This was also found to be the case in the present experiment. The growth rate of frog larvæ reared artificially is so variable, however, the writer lays no great stress upon this point.

The history of thyroidless Cultures 3 and 4 leads to the conclusion that 0.0000007666 and 0.0000003833 concentrations of elemental iodine are insufficient to induce complete metamorphosis in animals devoid of the thyroid apparatus, even when fed over fairly long periods of time. The organism is apparently unable to utilize the minute quantities of iodine absorbed through the integument without the aid of the thyroid mechanism to collect and store it, thus preventing its passage through the body unused. This is good evidence that larvæ with intact thyroid apparatus are capable of utilizing very much smaller quantities of elemental iodine than are thyroidless animals. The lower limit of the iodine concentration requisite for metamorphosis of normal larvæ was not determined, as 0.0000007666 and 0.0000003833 were the weakest used and both proved to be above the lower limit just necessary to induce metamorphosis. On the other hand, these same concentrations were too low to induce metamorphosis in thyroidless animals.

The determination of the exact amount of iodine absorbed by the

larvæ of any single culture during the course of the experiment was not attempted. Anuran larvæ apparently do not swallow much water, but are constantly absorbing it through the skin and eliminating it through the kidneys, thus a constant stream of water flows through the organism. It is surprising how much water a 6 mm. tadpole will absorb in 24 hours. Recently the writer[5] extirpated the kidneys of tadpoles, eliminating the excretory function. The result was a generalized body edema of enormous proportions due to water absorption. It is very unlikely that inorganic iodine will be absorbed by the larvæ at the same rate as water.

Iodine has a much wider distribution in nature than is commonly supposed, and is not confined solely to marine waters and organisms, and the thyroid glands of vertebrates. This halogen in some form or other, and in small quantities, is present in most surface water, and practically all organic matter, provided a fair sized sample is analyzed. It is surprising how many common foodstuffs contain traces of iodine. The amounts are of course very small, and this fact has been the chief hindrance in the way of recognizing iodine as of common occurrence, for it is only within recent years that a method sufficiently delicate to detect very minute traces of this substance has been devised. With further refinement of technique it is safe to assume that iodine will be found to have a far wider distribution in nature, and to play a greater functional part in organisms than is at present conceded.

In marine organisms, both animal and plant, iodine is usually found in some form, and certain animals like coral are said to have a specific iodine metabolism. Among land vertebrates, iodine is not only found in the thyroid, but normally occurs in the blood and tissues. For instance, Kendall[6] finds that the average iodine content of calves' blood is approximately 0.015 mg. per 100 cc. of blood. The amount in the tissues is somewhat greater, and in the liver is still greater than in the tissues. Other investigators have at various times reported the presence of iodine in the ovaries, adrenals, spleen, and salivary glands. The thyroids of infants a few weeks after birth are said to contain iodine, hence it is not improbable that human milk contains traces of this substance.

[5] Swingle, W. W., *J. Gen. Physiol.*, 1918–19, i, 509. McClure, C. F. W., *ibid.*, 261.
[6] Kendall, E. C., *Am. J. Physiol.*, 1919, xlix, 136.

There can be little doubt that the iodine of the thyroid is obtained by the organism from the food and water. Marine and Rogoff[7] have clearly shown that the affinity of thyroid tissue for iodine is so great that practically all iodine injected into the organism by experimental methods is "fixed" in the thyroid within a few minutes. Their experiments explain why it is that considerably more iodine is required to induce metamorphosis in thyroidless frog larvæ than in animals possessing glands.

The importance of iodine in the economy of the organism is at present little understood and much underestimated. The writer holds the view that this halogen will eventually take rank along with chlorine, phosphorous, and other elements essential to the maintenance of normal metabolism, growth, and development. The almost universal occurrence of a thyroid mechanism among the vertebrates for the assimilation and utilization of iodine in minute quantities, points to the conclusion that there is a definite iodine metabolism in these forms, and that it is necessary for the normal functioning of vital processes.

That elemental iodine functions within the thyroidless organism as such, is highly improbable; it is far more likely that it is combined with other substances in the blood and tissues, though what these substances are is unknown, just as it is unknown how the presence of the thyroid affects the iodine. In either case, however, the result is the same in frog larvæ; *i.e.*, normal metamorphosis. The fact that more iodine is required to bring about metamorphosis in thyroidless animals than in normal ones may indicate either that the thyroid gland tissue has added something to the iodine, thereby increasing its physiological activity, or that the gland has functioned as a storage organ and fixed the iodine in greater quantities, thus preventing its passage through the organism unused. It may well be that both these possibilities are realized in normal animals with intact glands. It is to this latter view that the writer adheres.

A recent writer[8] has stated that iodine has nothing to do with limb development in the larvæ of Urodela, and doubt is expressed that con-

[7] Marine, D., and Rogoff, J. M., *J. Pharm. and Exp. Therap.*, 1916, viii, 439.
[8] Uhlenhuth, E., *J. Gen. Physiol.*, 1918–19, i, 534.

ditions are different in anuran tadpoles. This writer says in this regard:

"It is remarkable, however, that in two so closely related groups of animals as the caudate and tailless batrachians, the same process should be caused by so different a mechanism. It is difficult to refute the suspicion that in the *Salientia* the development of the limbs may be initiated only indirectly by the iodine action, perhaps on account of some incidental anatomical structure which first must be broken down by the autolytic action of the iodine."

This statement should be of interest to students of anuran metamorphosis who have experimented with iodine, as it is a denial of the process, namely limb development, most obviously brought out by iodine administration in these forms. There can be no question that iodine has little or no effect upon limb development of urodeles, but also there can be no doubt that this same substance stimulates to a remarkable degree limb growth and formation in the Anura. Despite the close phylogenetic relationship between Urodela and Anura there exist some sharp distinctions between the larvæ of the two groups in regard to physiological responses to environmental conditions, and in developmental history. Metamorphosis in the Apoda and Urodela is restricted chiefly to the reduction of the gills, the closing of the clefts, and the loss of the gill chamber and the finny margin of the tail; but in frogs and toads, the change from the tadpole to the final form is a fundamental reorganization of the organism, involving practically every organ. The following quotation is from Boulenger:[3]

"In the newts the transformation is much more gradual (*i.e.*, than the metamorphic change in Anurans) without the intercalation of a tadpole stage. The final transformation in the Urodeles consists essentially in the loss of the gills and the development of the eye-lids. I may mention that although the larvæ of Urodeles are often called tadpoles, the term as applied to them is quite misleading, the larvæ of frogs having an altogether different form and organization."

Limb development in amphibians varies considerably in the different types. Thus, in most Anura, the limbs develop slowly and require months and years (*Rana catesbiana*) for complete development. In other forms, such for instance as the urodele, *Salamandra maculosa*, the young are born with legs.

Uhlenhuth regards gill reduction and shedding of the skin in urodeles as constituting metamorphosis. It is evident that such a con-

ception is not sufficiently broad to account for the fundamental changes incident to anuran transformation. For in these animals metamorphosis usually implies complete shedding of the tadpole characters, such as the fish-like tail, larval mouth, gills, intestinal hystolysis, and reorganization and development of the frog character by growth of limbs and other fundamental changes. Whether or not the autolytic action of iodine is responsible for all these body changes can only be determined by future investigation.

There are unquestionably other unknown chemical factors involved in the degenerative and regenerative processes involved in metamorphosis, and as investigation in this field has scarcely begun, all generalizations should be regarded as premature.

In conclusion the writer wishes to acknowledge his indebtedness to Professor E. N. Harvey and Professor E. G. Conklin for valuable criticism and advice.

INFLUENCE OF THE CONCENTRATION OF ELECTROLYTES ON THE ELECTRIFICATION AND THE RATE OF DIFFUSION OF WATER THROUGH COLLODION MEMBRANES.

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I. Influence of the Concentration of Electrolytes upon the Rate of Diffusion of Positively Charged Particles of Water through Collodion Membranes.

When we fill a collodion flask, as described in a preceding publication,[1] with a solution of a non-electrolyte, *e.g.* cane sugar, grape sugar, or glycerol, and dip the flask into a beaker containing distilled water, the level of the liquid in the flask will rise, as is to be expected on the basis of the gas pressure theory of osmosis. When we close the opening of the collodion flask with a rubber stopper, perforated by a glass tube with a bore of about 2 mm. in diameter serving as manometer, the rate of diffusion of water into the solution can be conveniently followed. Since at the same time sugar will diffuse out of the flask into the surrounding distilled water, the rise of the column of liquid in the manometer will cease after some time (*e.g.* after about 70 minutes at 24°C.) and will be followed by a fall in the level of the liquid, until finally the solutions inside and outside the collodion flask become identical. It is therefore necessary to consider only the initial rise of liquid in the manometer as an indicator for the attractive action of the solution upon water, and in all the figures given in this paper the readings were made 20 minutes after commencement of the experiment. At the beginning of the experiment the level in the manometer was usually about 30 mm. above that of the distilled water in the beaker in order to discover possible cases of negative osmosis. All collodion flasks had practically the same sur-

[1] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 717.

face, being cast inside of the same Erlenmeyer flask, and during the experiment the whole area of the flask was submersed in the beaker. The temperature was always 24°C.

In Fig. 1 the ordinates are the values for the rise in the level of the solution in the glass tube (after the first 20 minutes) which occurred when the collodion flasks filled with different concentrations of cane sugar, grape sugar, or glycerol were dipped into beakers containing distilled water. The abscissæ are the logarithms of the concentration of the sugar solution. The reader will notice that for concentrations below M/64 the curves run practically parallel to the base line while a sharp rise begins at about M/16 or M/8. If the rise is plotted over the concentration (instead of over the logarithms of the concentration) the curve is almost a straight line between concentrations of M/32 and 1 M (Fig. 2), as was to be expected on the basis of the gas pressure theory. When, however, we make the same experiments with solutions of *electrolytes*, separating them from pure water by collodion membranes, a curious phenomenon is observed, which was partly described in the first paper; namely, that at a very low concentration of electrolyte the rate of diffusion of water through the collodion membrane from pure solvent into the solution increases rapidly with increasing concentration and that it reaches a maximum at a comparatively low concentration[1] of the electrolyte. It is easier to follow the facts to be described with the help of the curves given in Fig. 3. The abscissæ are the logarithms of the concentration of the solution, while the ordinates give the height to which the liquid in the manometer has risen in 20 minutes. The reader will notice that the curves for five sodium salts are given—NaCl, Na_2SO_4, Na_2 oxalate, Na_3 citrate, $Na_4Fe(CN)_6$. The solutions must not be acid for this experiment, and the hydrogen ion concentration of the solutions of NaCl, Na_2SO_4, and Na_2 oxalate was almost that of the point of neutrality (pH about 6.0, or between 6.0 and 7.0), while the two other solutions were slightly alkaline. Beginning with the lower concentrations of the solutions of these salts the curves rise sharply with the increase in concentration, reach a maximum at a concentration of about M/256, and then with a further increase in concentration the curves fall abruptly to reach a minimum, varying for the different salts between M/32 and M/8. After this the curves rise again.

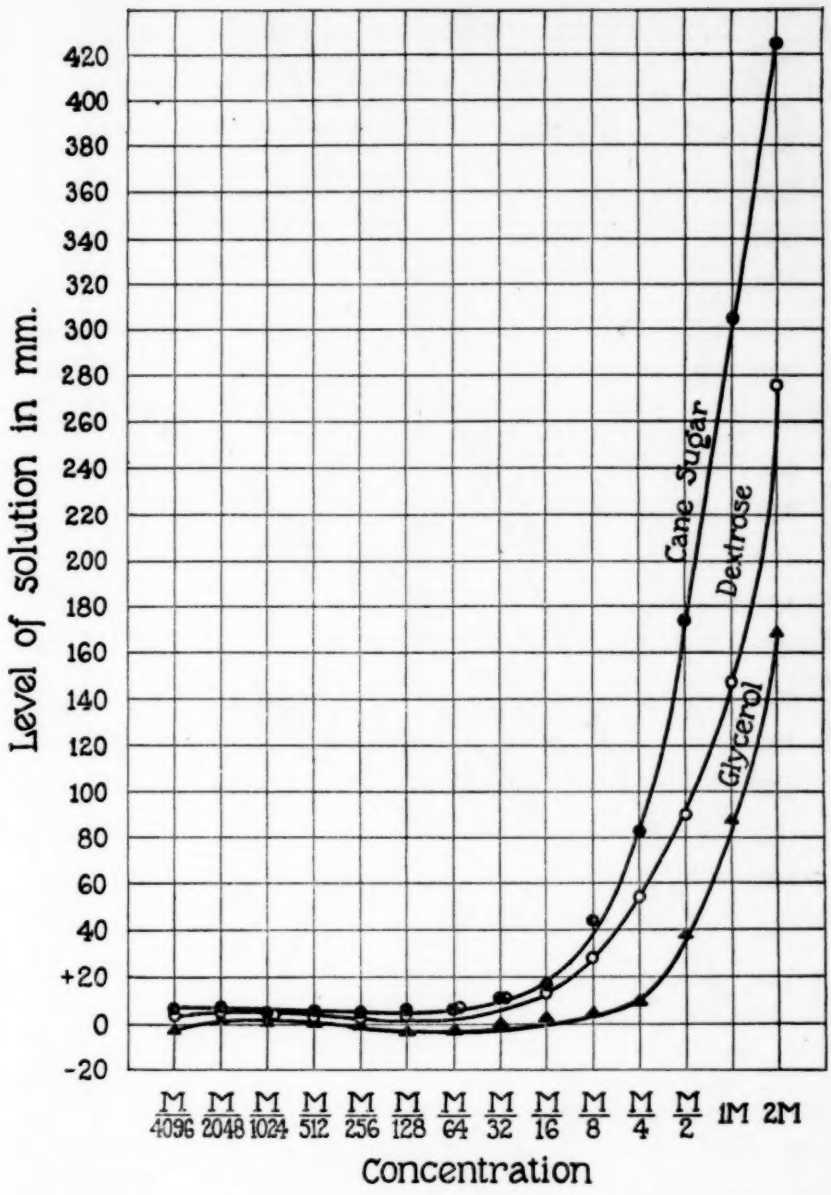

FIG. 1. Influence of concentration of non-electrolytes on initial rate of diffusion of water through a collodion membrane from pure solvent to solution. Abscissæ are logarithms of concentrations, ordinates the rise in mm. in level of solution in the manometer tube after 20 minutes.

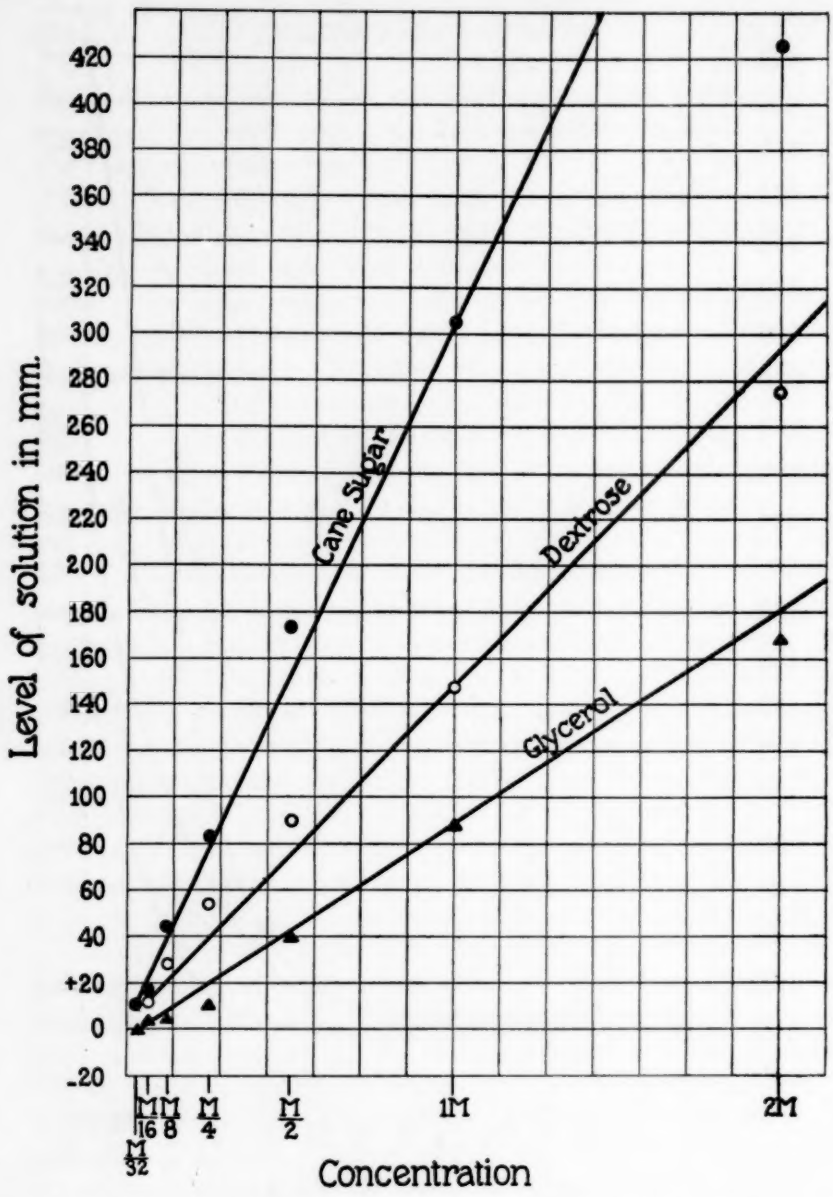

FIG. 2. The same as in Fig. 1, except that abscissæ in Fig. 2 are the concentrations instead of the logarithms of concentrations as in Fig. 1. Within concentrations of M/32 and 1 M the initial rise of liquid in 20 minutes is in direct proportion to the concentration, as van't Hoff's law demands.

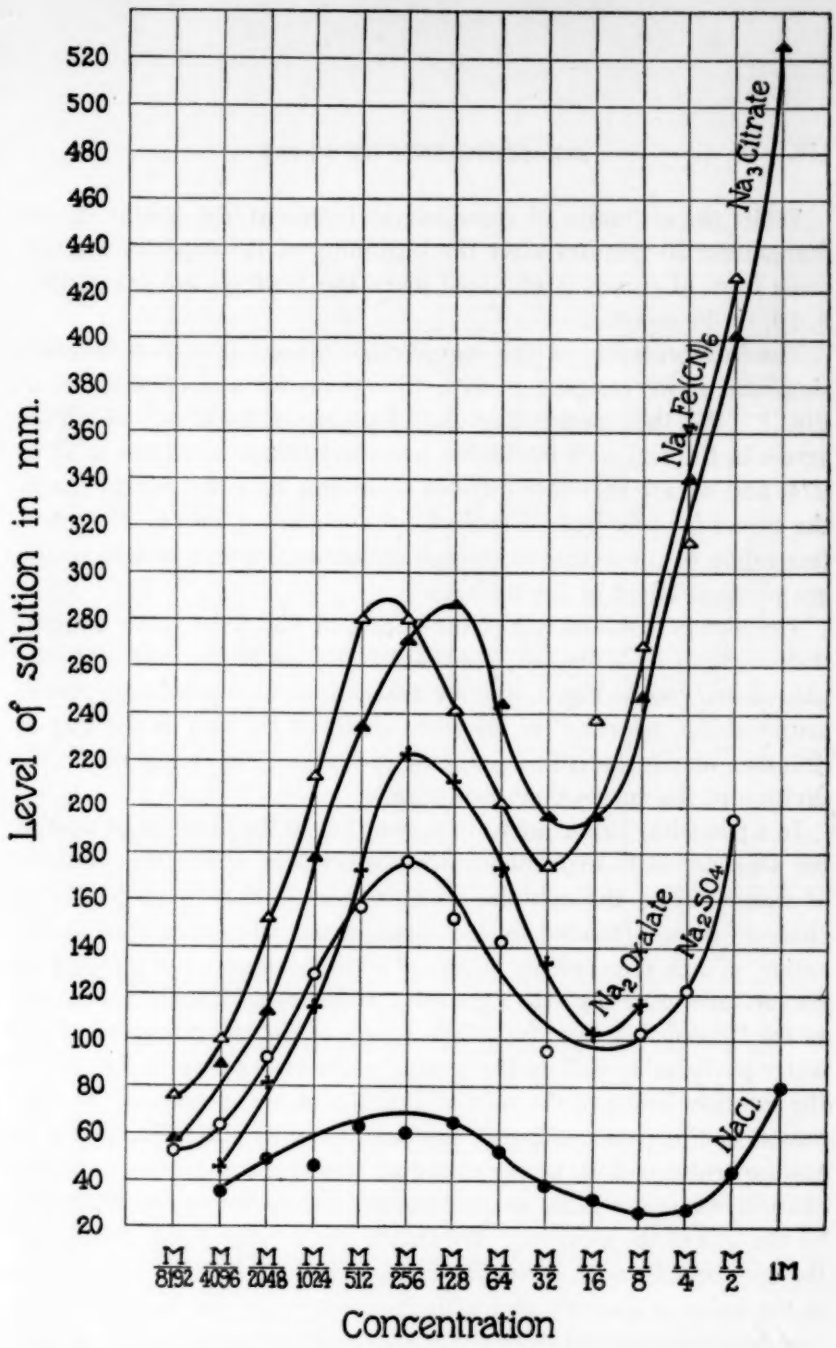

FIG. 3. Curves representing influence of concentration of five different sodium salts upon initial rate of diffusion of water through a collodion membrane from pure solvent to solution. Abscissæ are the logarithms of concentration. Ordinates represent level of solution in manometer tube after 20 minutes. The curves rise steeply until the concentration of M/256 is reached, then, with further increase in concentration, the curves drop, and rise again when the concentration is between M/16 and M/4.

While the ordinates of these curves represent the height in the manometer 20 minutes after the beginning of the experiments, the same form of curves is obtained when the readings are taken after 5, 10, or 30 minutes.

The interpretation of this complicated system of curves becomes simplified if we compare it with the curves for non-electrolytes in Fig. 1. We then notice that the character of both sets of curves agrees in the region of the higher concentrations above from M/32 to M/4, and we are therefore inclined to assume that the second rise in the curves for solutions of sodium salts beginning with M/32 to M/4 (according to the nature of the salt in solution) is due mainly to the gas pressure effect of the solution.

The abrupt rise and fall of the curves in Fig. 3 for lower concentrations than M/32 are not repeated in the case of the curves for the non-electrolytes in Fig. 1, and we are inclined to ascribe these peculiarities of the curves to the electrical action of the ions on the rate of diffusion of the electrified particles of water. Fig. 4 expresses this division of the curves diagrammatically.

In a preceding paper[1] we have shown that in the presence of neutral (or alkaline) salts with monovalent or bivalent cation the particles of water diffuse through the membrane as if they were positively charged, being attracted by the anion of the salt and repelled by the cation with a force which increases with the number of charges of the ion (and inversely with a quantity which we arbitrarily designated as the "radius" of the ion). The source of the electrification of the water particles as well as the specific mechanism by which the ions of the solution influence the rate of diffusion of water will not be discussed in this paper. It will, however, simplify the presentation of our experiments if it be permitted to describe our results as if the charged water particles were attracted or repelled electrostatically by the ions of the solution. We notice that in Fig. 3 the curves rise the more rapidly with increasing concentration the higher the valency of the anion of the salt, and with the reservation just mentioned we may ascribe this initial steep rise in the curves between a concentration of the solution of 0 to a concentration of M/256 to the fact that the attractive action of the anion of the sodium salts upon the rate of diffusion of the positively charged particles of water through the mem-

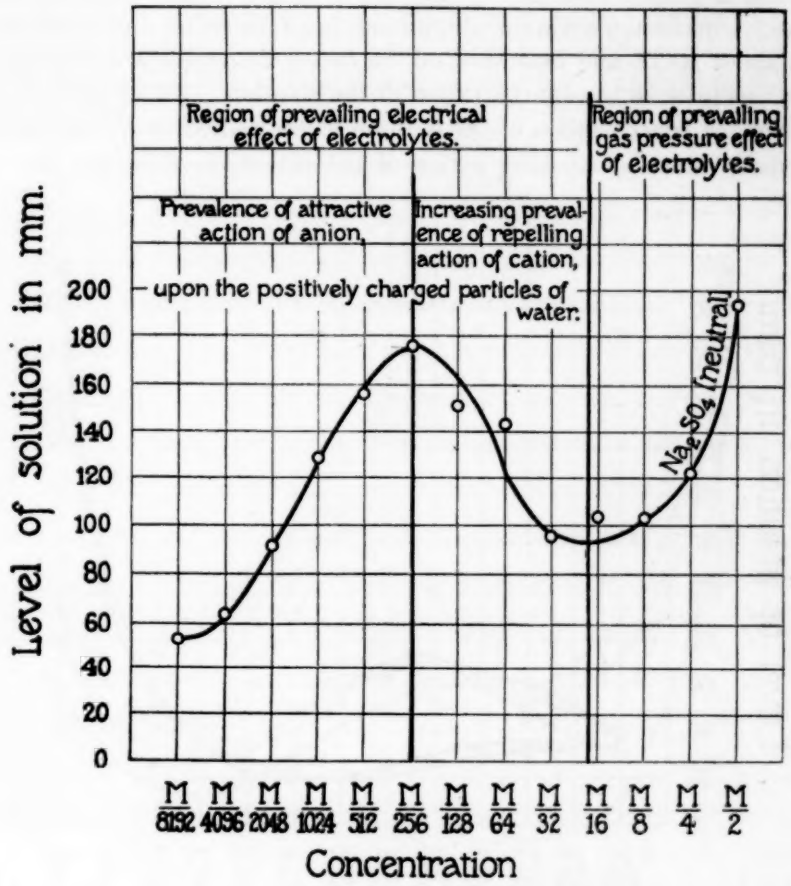

FIG. 4. Explanation of the curves in Fig. 3 given for the Na_2SO_4 curve. The first rise and fall between M/8,192 and M/16 represent the electrostatic action of ions of the solute upon the rate of diffusion of positively charged water particles. The second rise beyond M/16 represents the rise due to the gas pressure effect of the solute. The steep rise of the curve between 0 and M/256 is due to the prevalence of the attractive action of anion upon the positively charged molecules of water, while the drop beyond M/256 is due to the fact that the repelling action of cation upon the positively charged particles of water increases more rapidly with increasing concentration than the attractive action of SO_4 for the water.

brane is greater than the repelling action of the cation. The curves reach a maximum when the solution reaches a molecular concentration of about M/256 and from now on the curves drop rapidly with a further increase in the concentration of the solution. We assume that when the concentration of the solution of the sodium salts reaches a certain value the repelling action of the cation—in this case Na—

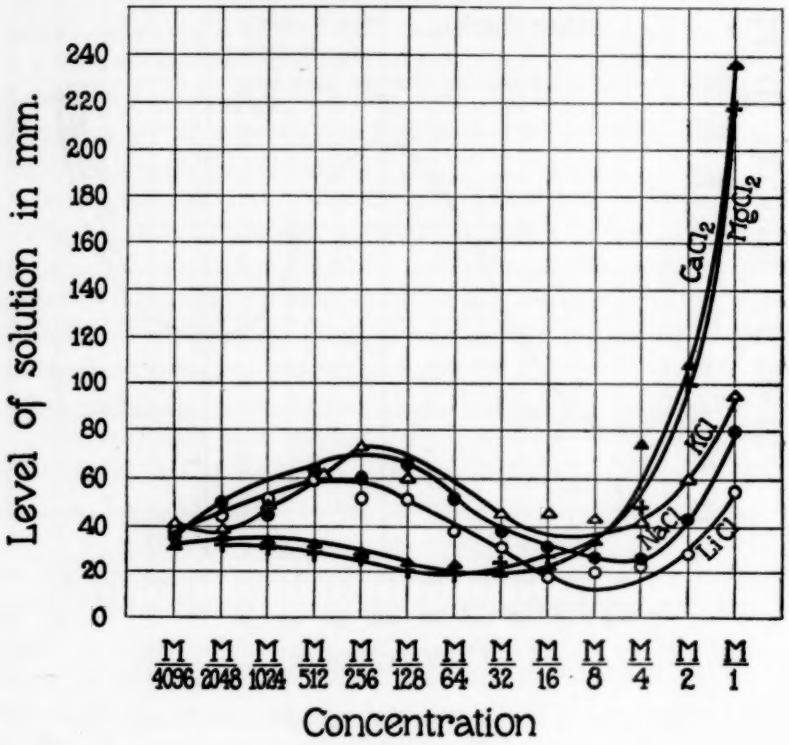

FIG. 5.

increases more rapidly with increasing concentration than the attractive action of the anion upon the positively charged particles of water. This drop, however, is comparatively smaller the higher the valency of the anion. In the case of sodium chloride (Fig. 3) the curve is lower at M/8 (the minimum) than at M/4,096, while for SO_4 and oxalate the minimum reached by the curves (at M/16) is considerably above that

reached by the NaCl curve, and the minimum for the citrate and ferrocyanide is still higher. This was to be expected from the fact that the attractive action of the anion upon the positively charged particles of water is higher the greater the number of charges or the valency of the anion.

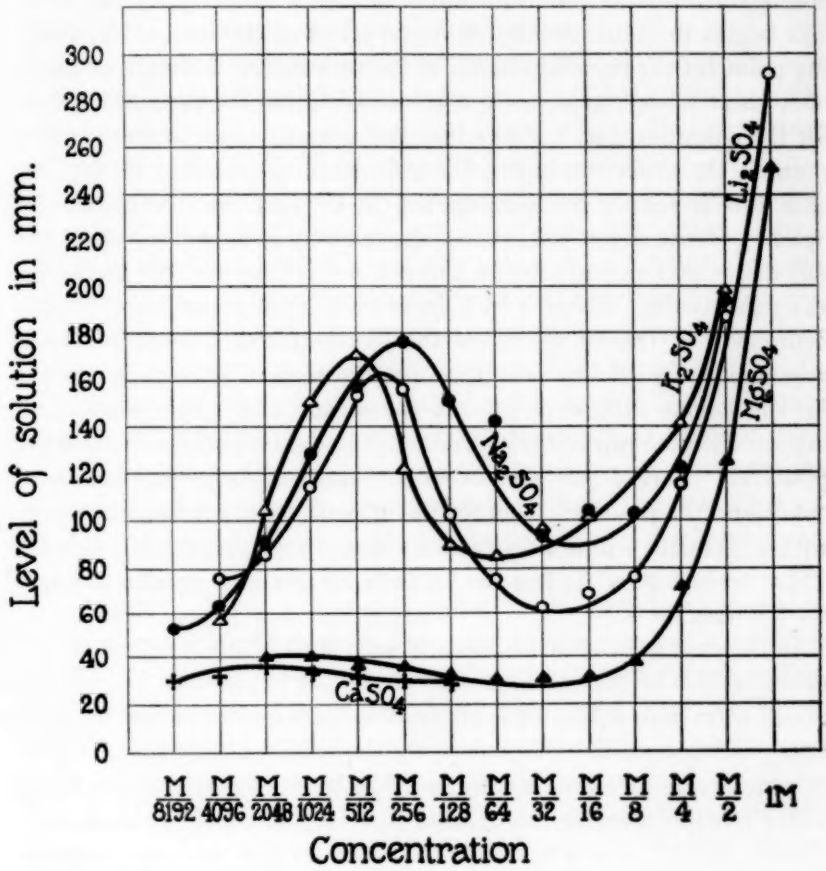

FIG. 6.

FIGS. 5 and 6. Curves representing influence of different chlorides (Fig. 5) and sulfates (Fig. 6) on initial rate of diffusion of water from pure solvent into solution, showing that the drop is greater in case of Li than of Na, or of K, and supporting the view that the drop is due to the repelling effect of the cation upon the positively charged particles of water.

The facts given are not peculiar to sodium salts but can be demonstrated in the case of all neutral or alkaline solutions of salts with monovalent or bivalent cation. Fig. 5 gives the curves for different chlorides and Fig. 6 for different sulfates. All the curves for these salts rise up to a concentration of about M/256 and then fall and finally rise again when the concentration is so high that the gas pressure effect begins to obliterate the electrical effect of the ions. The turning point for the commencement of the prevalence of the gas pressure effect is at a lower gram-molecular concentration for the sulfates than for the chlorides; Fig. 3 shows that the concentration of the turning point is the lower the higher the valency of the anion. It may be that this is simply the consequence of the fact that the higher the valency of the anion the greater the number of ions into which the salt dissociates electrolytically, but it is not yet certain that this is the full explanation. Another fact, however, is certain; namely, that the depression of the curves when the concentration exceeds M/256 is greater for the lithium salts than for the sodium salts, and greater for the sodium salts than for the potassium salts. This was to be expected if the drop is due to the repelling action of the cation on the positively charged particles of water, since the repelling action of cations on the positively charged particles of water increases inversely with a quantity which we arbitrarily designate as the "ionic radius." This "ionic radius" is less for Li than for Na and presumably less for Na than for K.

In the case of neutral solutions of salts with bivalent cation the repelling action of the cation is so great that it balances or exceeds the initial attractive action of a bivalent or monovalent anion for positively charged particles of water; the depressing action of the cation in concentrations above M/256 upon positively charged particles of water remains, however, noticeable especially in the SO_4 curves.

II. Influence of the Concentration of Electrolytes upon the Rate of Diffusion of Negatively Charged Particles of Water through Collodion Membranes.

The curves given in Figs. 3 to 6 contain a paradoxical fact, which can be expressed as follows: *when solutions of neutral or alkaline salts with monovalent or bivalent cation are separated by a collodion membrane*

from pure water the attraction of the solution for water diminishes with increasing concentration for concentrations between M/256 *to about* M/32 *or above, according to the nature of the electrolyte.* This is exactly the reverse of what we should expect on the basis of van't Hoff's law which demands that the attraction of the solution for water should increase with the concentration. Such a reversal occurs only in the case of electrolytes and we assume provisionally that it is due to the fact that the repelling action of the cation of the electrolyte upon the positively charged particles of water increases inside the critical range of concentrations more rapidly with the concentration than the attractive action of the anion upon the same particles of water.

It will be necessary to test this assumption by further experiments.

We have shown in the first paper that water particles diffuse through collodion membranes in the form of *positively* charged particles from pure solvent to solutions of electrolytes with monovalent or bivalent cation when the solution is neutral or alkaline in reaction. When, however, the solution is rendered sufficiently acid the water particles diffuse through the membrane as if they were *negatively* charged, being attracted by the cation and repelled by the anion of the electrolyte with a force increasing with the valency of the ions. We will now consider the influence of the concentration of electrolytes upon the rate of diffusion of *negatively* charged particles of water through the membrane.

We have stated in a former paper[2] that the collodion membranes were suspended for 1 night in a 1 per cent gelatin solution before being used; after this they were used sometimes for a week or more without any further gelatin treatment. The membranes were, of course, rinsed a large number of times in warm water after the gelatin treatment to remove all the gelatin that could be removed by rinsing. This gelatin treatment is not necessary to obtain the results with neutral solutions of electrolytes described in the preceding chapter. The gelatin treatment of the membrane is, however, necessary to obtain the results with negatively charged particles of water to be described in this chapter. We intend to return to this fact in a later paper.

[2] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 87.

Negatively charged particles of water are attracted powerfully by bivalent or polyvalent cations and repelled powerfully by bivalent or polyvalent anions. We may, therefore, expect that when we make a $CaCl_2$ solution sufficiently acid through the addition of HNO_3 or HCl the curves showing the influence of the concentration of the electrolyte on the rate of diffusion of water should be similar to the curves in Figs. 3, 4, and 6, representing the influence of different concentrations of neutral solutions of Na_2SO_4 upon the rate of diffusion of positively charged particles of water through the same membranes. Fig. 7 shows that this is correct. The two upper curves represent the influence of variations in the concentration of $CaCl_2$ or $Ca(NO_3)_2$ dissolved in M/1,024 or M/128 HNO_3 or HCl. These acid solutions of $CaCl_2$ or $Ca(NO_3)_2$ cause a negative electrification of the water particles diffusing through the membrane. The attraction of the negatively charged water particles by Ca ions increases with an increase in the concentration of the calcium salt up to M/64, when the curve falls until the concentration of the calcium salt is M/4 where a minimum is reached. After this the gas pressure effect makes itself felt and the curve rises rapidly with rising concentration. In this case the drop in the two curves for the acid solutions when the concentration of the calcium salt exceeds M/64 is due to the fact that beyond a certain concentration of the calcium salt the repelling effect of the anion (Cl and NO_3) upon the negatively charged particles of water increases more rapidly with increasing concentration than the attractive action of the Ca ion.

The fact that the curve for $Ca(NO_3)_2$ in M/128 HCl is slightly lower than the curve for $CaCl_2$ in M/1,024 HNO_3 is due to the "negative osmosis" caused by acid solutions of sufficient concentration, as will be shown in the next chapter.

The three lower curves of Fig. 7 represent the influence of various concentrations of $CaCl_2$ upon positively charged particles of water and these curves are similar to those given for the effect of neutral solutions of $CaCl_2$ in Figs. 5 and 6. When $CaCl_2$ is dissolved in M/128 KOH the rise and drop of the curve become a little more marked than when the solution of $CaCl_2$ is neutral. The reason for this will become clear through the discussion in the next chapter of the phenomenon of negative osmosis caused by $Ca(OH)_2$.

When we render Na_2SO_4 solutions acid (by dissolving the salt in M/128 or M/1,024 HNO_3) the negatively charged particles of water diffusing through the membrane will be repelled by the SO_4 ion and attracted by both the Na and H ions. Since the SO_4 ion carries two

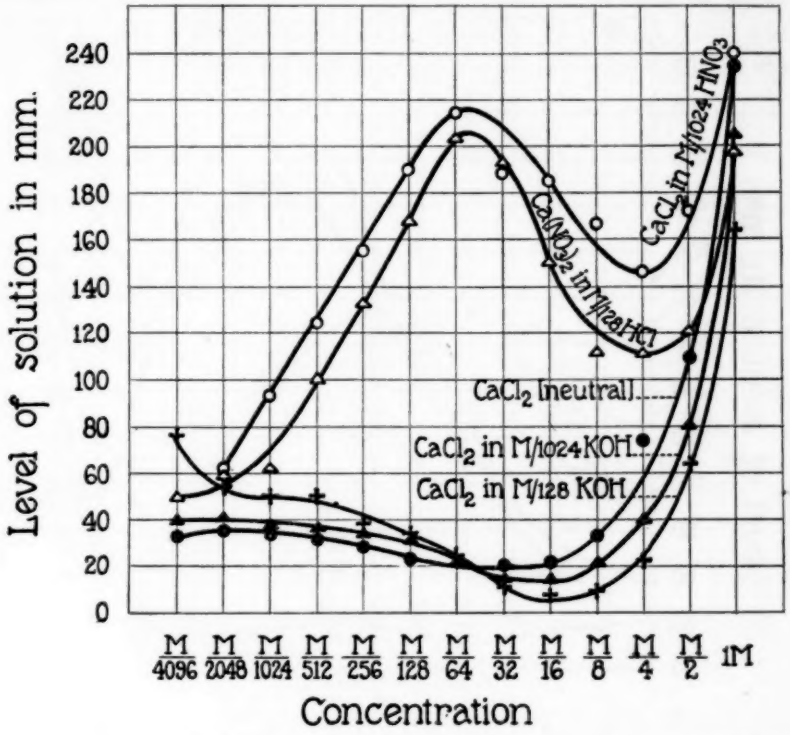

FIG. 7. Upper two curves representing influence of acid solutions of $CaCl_2$ or of $Ca(NO_3)_2$ upon negatively charged particles of water. At first there is a steep rise of the curve due to prevalence of attraction of Ca for the negatively charged particles of water, then beyond M/64 a drop in the curve due to prevalence of repelling action of NO_3 and Cl upon the negatively charged particles of water. No initial rise in the three lower curves, since in neutral and alkaline solutions of Ca water diffuses in the form of positively charged particles which are too strongly repelled by Ca to permit an initial rise in the level of the solution.

charges while the cations Na and H are monovalent the greater electrostatic action of the SO_4 ion should inhibit the electrostatic attraction of water by such solutions in the same way as is done by neutral so-

lutions of $CaCl_2$ in the case of positively charged particles of water. Fig. 8 shows that this is the case. The two lower curves in Fig. 8 show the influence of concentrations of sufficiently acid solutions of Na_2SO_4 upon the rate of diffusion of water from distilled water into

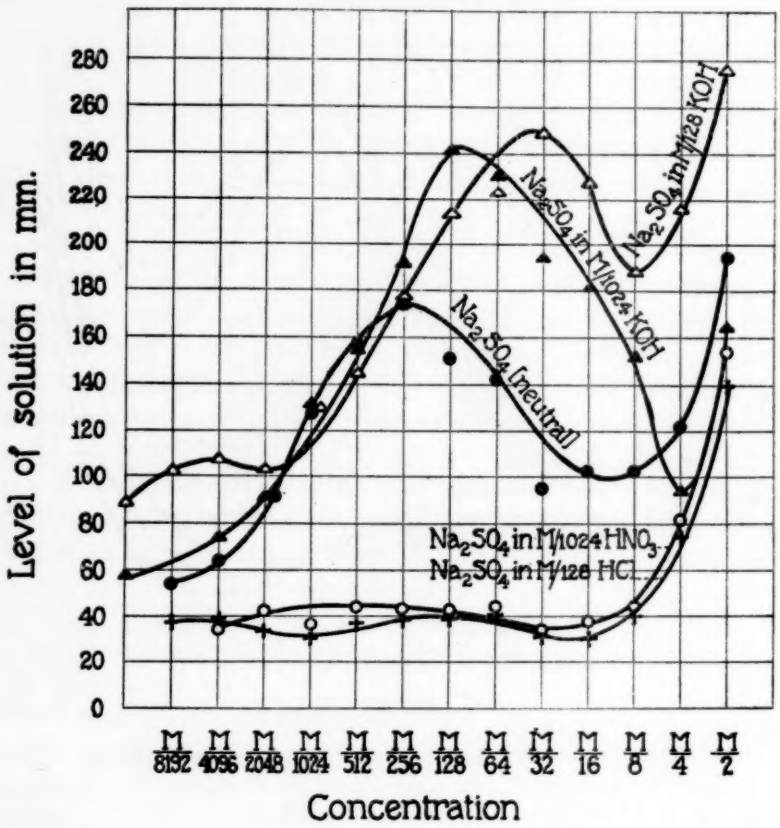

FIG. 8. The curves for solutions of Na_2SO_4 when sufficiently acid show no initial rise, since the negatively charged particles of water are too strongly repelled by the SO_4 ions.

solution. The curves resemble the curves for the effect of different concentrations of non-electrolytes, except for an intimation of a slight drop for concentrations of Na_2SO_4 between M/64 and M/8. The three upper curves represent the influence of concentrations of different

neutral and alkaline solutions of Na_2SO_4 upon the rate of diffusion of positively charged water particles.

It was finally expected that the curves representing the influence of various concentrations of NaCl upon the rate of diffusion of water through collodion membranes should be similar in neutral, acid, and alkali solutions, for the reason that anion and cation of the salt are

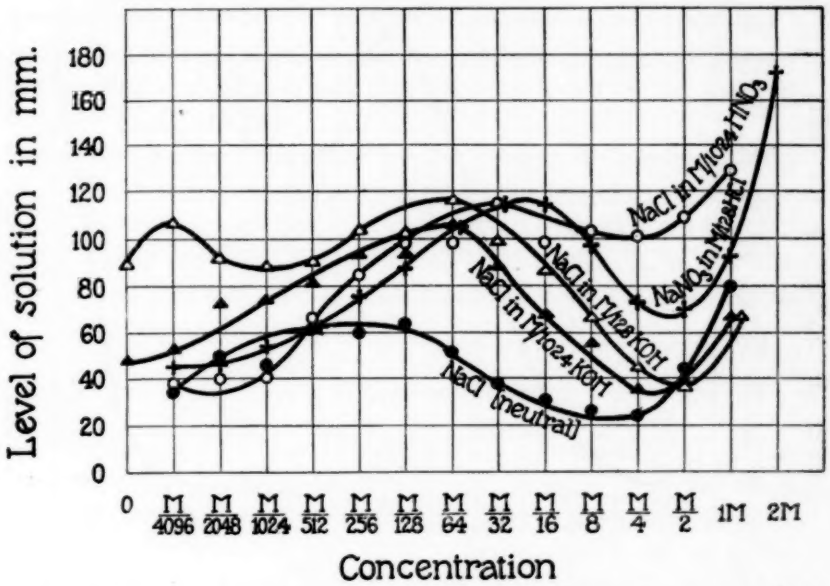

FIG. 9. Salts with monovalent anion and cation, like NaCl, show both rise and drop in alkaline, neutral, and acid solutions. In neutral and alkaline solutions the first rise is due to the attractive action of the Cl ion and the drop to the repelling action of the Na ion upon the positively charged particles of water. In acid solution the first rise in the curve is due to the attractive action of the Na ion and the drop to the repelling action of the Cl ion upon the negatively charged particles of water.

both monovalent (Fig. 9). In the presence of a sufficiently acid solution of NaCl the particles of water diffuse as negatively charged molecules through the membrane which are attracted by the Na ions and repelled by the Cl ions. The initial rise of the curve is due to the attractive action of the Na ion, and the drop in the curve is due to the increasing prevalence of the repelling action of the Cl ion. In neutral

and alkaline solutions of NaCl the Cl ion attracts the water (which is charged positively) and the fall of the curve is due to the Na ion. The complete suppression of the rise which is caused in $CaCl_2$ when the solution is neutral or alkaline, and in Na_2SO_4 when the solution is acid, is due to the fact that in these cases the attracting ion is mono-

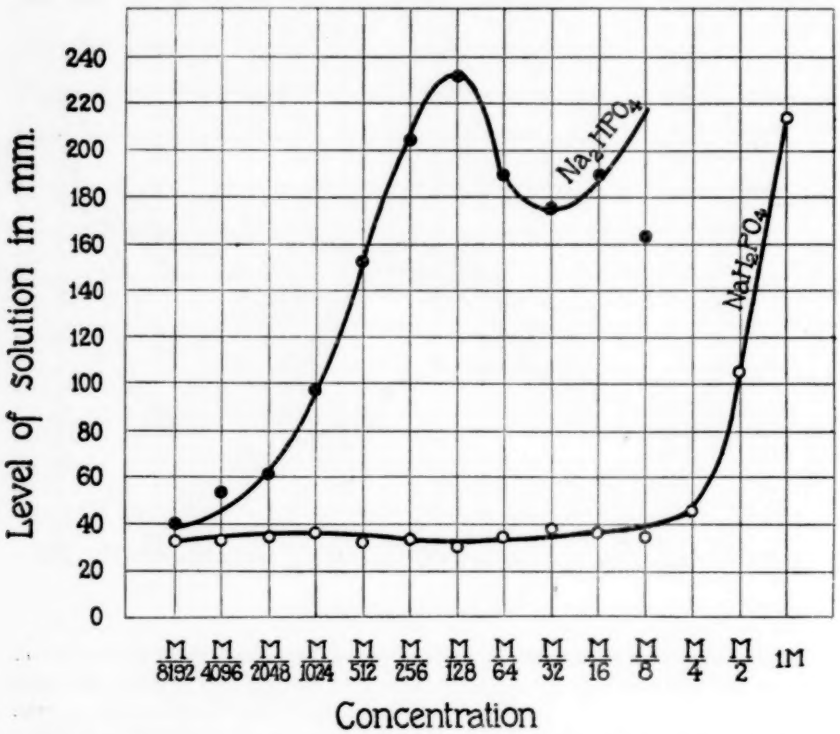

FIG. 10. The Na_2HPO_4 curve shows the initial rise and drop since the water diffuses in the presence of this salt in the form of positively charged particles which are attracted by the trivalent PO_4 ion and repelled by the monovalent Na and H ions. The NaH_2PO_4 curve shows no initial rise, since in the presence of this salt water diffuses in the form of negatively charged particles which are repelled by the trivalent PO_4 ion and attracted by the monovalent Na and H ion.

valent and the repelling ion bivalent. Fig. 9 shows that the curves representing the influence of the concentration of NaCl solutions upon the rate of diffusion of water are similar for neutral, alkaline, and acid solutions of NaCl. The drop is more considerable for $NaNO_3$ solu-

tions in M/128 HCl than in M/1,024 acid, on account of the negative osmosis caused by solutions of acids, as the next chapter will show.

Fig. 10 shows the difference in the influence of concentration upon the rate of diffusion of water in the case of Na_2HPO_4 and NaH_2PO_4. In the presence of the latter solution the water diffusing through the membrane is negatively charged and the curve does not show the initial rise and drop but shows only the gas pressure effect of the solution. In the case of Na_2HPO_4 water diffuses in the form of positively charged particles and the curve shows the rise and drop as described for neutral and alkaline salts in which the anion has a higher valency than the cation.

We can therefore state that for the diffusion of negatively as well as positively electrified particles of water the law of van't Hoff is reversed within a certain range of concentrations of electrolyte—in the extreme between about M/256 and M/4—inasmuch as in this range the attraction of a solution of an electrolyte for pure water diminishes with increasing concentration.

III. Negative Osmosis.

Dutrochet,[3] Graham,[4] and Flusin[5] have observed a striking phenomenon of negative osmosis in certain acids; namely, that the stream of liquid diffuses through pig's bladder from acid to pure water, instead of in the opposite direction. This was observed for tartaric and oxalic acids. Flusin tries to explain the phenomenon on the basis of a difference in degree of imbibition or swelling of the two sides of pig's bladder, one side being in contact with pure water where the swelling is slight, the other being in contact with acid where the swelling is great. Bartell, and Bartell and Hocker[6] have observed negative osmosis through porcelain even in the case of some neutral

[3] Dutrochet, H., *Ann. chim. et. phys.* 1835, lx, 337.

[4] Graham, T., *Phil. Tr.*, 1854, cxliv, 177.

[5] Flusin, G., *Ann. chim. et phys.*, 1908, xiii, 480. For a discussion of the literature see Höber, R., Physikalische Chemie der Zelle und der Gewebe, Leipsic, 4th edition, 1914, 248.

[6] Bartell, F. E., *J. Am. Chem. Soc.*, 1914, xxxvi, 646. Bartell, F. E., and Hocker, C. D., *J. Am. Chem. Soc.*, 1916, xxxviii, 1029, 1036.

salts. The writer has never observed negative osmosis when solutions of neutral salts were separated from pure solvent by collodion membranes.

The writer has recently investigated the influence of the concentration in acids and alkalies on the rate of diffusion of water through collodion membranes (previously treated with gelatin), with the result that the phenomenon of negative osmosis described for tartaric and oxalic acids is very common in acids and alkalies, that it *occurs in exactly the same range of concentration where the drop in the curves of the neutral salt solutions occurs, namely within a range between* M/256 *to about* M/4, *and that the phenomenon is in reality nothing else but this drop.* The difference between the nature of the drop in the case of solutions of neutral salts on the one hand and of solutions of acids and alkalies on the other is that in the case of alkalies and acids the drop is not only relative but absolute. Titration experiments show that acid diffuses from the acid solution into distilled water and that the concentration of acid in the solution is considerably less after 20 minutes than at the beginning. Since at the same time the total volume of solution diminishes—this being the nature of negative osmosis—we must conclude that the diminution of the volume of the solution is due to the fact that the combined volume of acid and water diffusing out from the solution is slightly larger than the volume of water diffusing simultaneously into the solution. We shall return to this problem in a subsequent paper. Since acids as well as bases diffuse into the pure solvent the phenomenon of negative osmosis can only be observed during a short period at the beginning of the experiment.

In all the experiments described in the preceding pages it was necessary to put the solution into the collodion flask and to dip the latter into distilled water in order to observe the influence of the solution on the initial rate of diffusion of water. More water diffuses into the solution than diffuses simultaneously in the opposite direction and as a consequence the level of the water in the glass tube rises. When we put solutions of certain acids or alkalies into the collodion flasks and dip these flasks into distilled water the level of the liquid in the manometer falls instead of rising. If, however, we put the solution of the same acids or alkalies outside, filling the collodion flask with

distilled water, we observe an initial rise of liquid in the manometer. For these experiments with acids, the collodion membranes must be bathed once over night in a 1 per cent gelatin solution. Fig. 11 shows the influence of the nature and concentration of acid upon the phenomenon. Since we are dealing here with a loss of volume on the

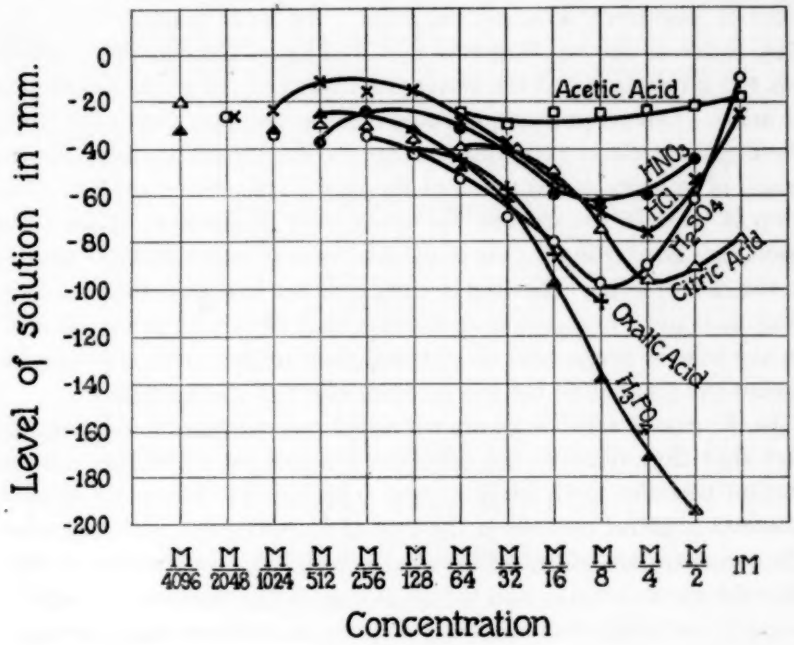

FIG. 11. Negative osmosis in acids, showing that the drop occurs here as in all other cases in a range of concentrations between M/256 and M/8 or M/4. The drop is greater for acids with bivalent or trivalent anion than for acids with monovalent anion, showing that the drop is due to the repelling action of the anion upon the negatively charged particles of water.

part of the solution, we must plot the rise observed in the manometer as a negative quantity below the zero line. The curves show that the drop is confined to the same range of concentrations where a similar drop occurs in the case of the neutral salts. The level of the water in the manometer tube was at the beginning of the experiment about 30 mm. When the flasks thus filled with distilled water were put

into beakers containing solutions of acid the level of the pure water in the flask rose during the first 20 minutes or more whenever the concentration of the acid was M/256 and the rise increased with an increase in the concentration until the concentration was about M/4. Then the level of the water inside the flask fell again.

In the presence of acids water diffuses through the membrane in the form of negatively charged particles. We have assumed that the drop is due to the repelling action of that ion of the electrolyte which has the same charge as the water particles; *i.e.*, the anion in the case of acids. Our assumption is supported by the fact that aside from the degree of electrolytic dissociation the drop increases with the increase of valency of the anion of the acid as we should expect. The drop is least in the case of the acids with monovalent anion (NO_3 and Cl), is greater in the case of SO_4 and oxalate anion, and still greater in the case of PO_4. The drop in citric acid is a little less than in oxalic acid, as is to be expected from the fact that citric acid is a weak acid. In the case of acetic acid no rise and drop in the curve is noticeable except the rise due to the gas pressure effect of the solution.

In the case of alkalies we are restricted to low concentrations by the fact that they dissolve the collodion membranes when the concentration becomes moderately strong. As Fig. 12 shows, we observe absolute negative osmosis in the case of $Ca(OH)_2$ and $Ba(OH)_2$ when the concentration of the solution exceeds M/256. In the case of alkalies with monovalent cation the usual drop in the curves—*i.e.* relative negative osmosis—was observed, but not an absolute negative osmosis; *i.e.*, an absolute diminution of the volume of the solution.

The negative osmosis in the case of acids and alkalies becomes absolute for the reason that the initial rise in the curve due to the attractive action of the ion with the opposite sign of charge from that of the water is too slight or entirely lacking in the case of the acids or alkalies.

The writer was interested in finding out whether aluminium salts with bivalent or trivalent anion induce negative osmosis. In Fig. 13 are plotted the curves representing the influence of different concentrations of Al_2Cl_6, $Al_2(SO_4)_3$, and aluminium citrate on the rate of diffusion of water through a collodion membrane. All the solutions had about the same hydrogen ion concentration and the water diffused

through the membrane in the form of negatively charged molecules, which according to our theory are attracted by the Al ion and repelled by the anion, the repelling action increasing with the valency of the anion. The curves show that Al_2Cl_6 attracts water very violently, $Al_2(SO_4)_3$ much less, and that aluminium citrate acts like a solution of a non-electrolyte, the attractive and repelling action of the two trivalent ions, Al and citrate, balancing each other. But no negative osmosis was noticed. This shows that the electrostatic effect of the H ion upon the rate of diffusion of water is considerably smaller than

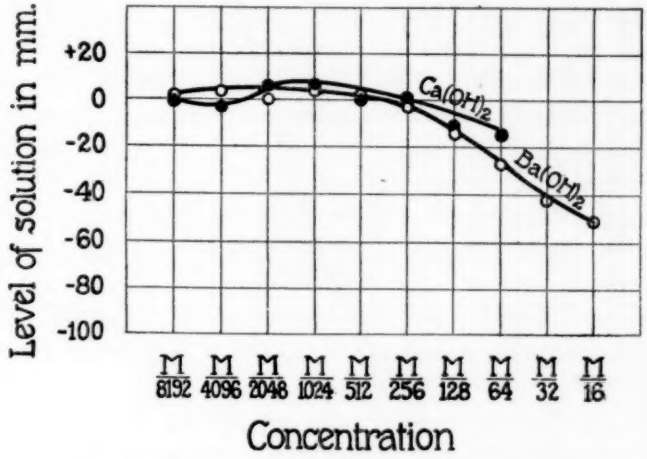

FIG. 12. Curves showing negative osmosis in the case of $Ca(OH)_2$ and $Ba(OH)_2$ occurring in that range of concentrations where the drop in Fig. 3 occurs.

the electrostatic action of trivalent cations like Al, while the electrifying effect of the H ion upon the water seems to be more nearly equal to the electrifying effect of the trivalent cation upon water. This suggests that the electrifying effect of ions upon water and their electrostatic effect upon the rate of diffusion of water are due to different qualities of the ion.

The curves for aluminium salts in Fig. 13 show no drop. As a matter of fact, a slight drop can be demonstrated in the $AlCl_3$ curves but in concentrations higher than those in Fig. 13, namely about M/32 or M/16, a phenomenon which finds its explanation perhaps in the fact that the cation is trivalent and the anion only monovalent.

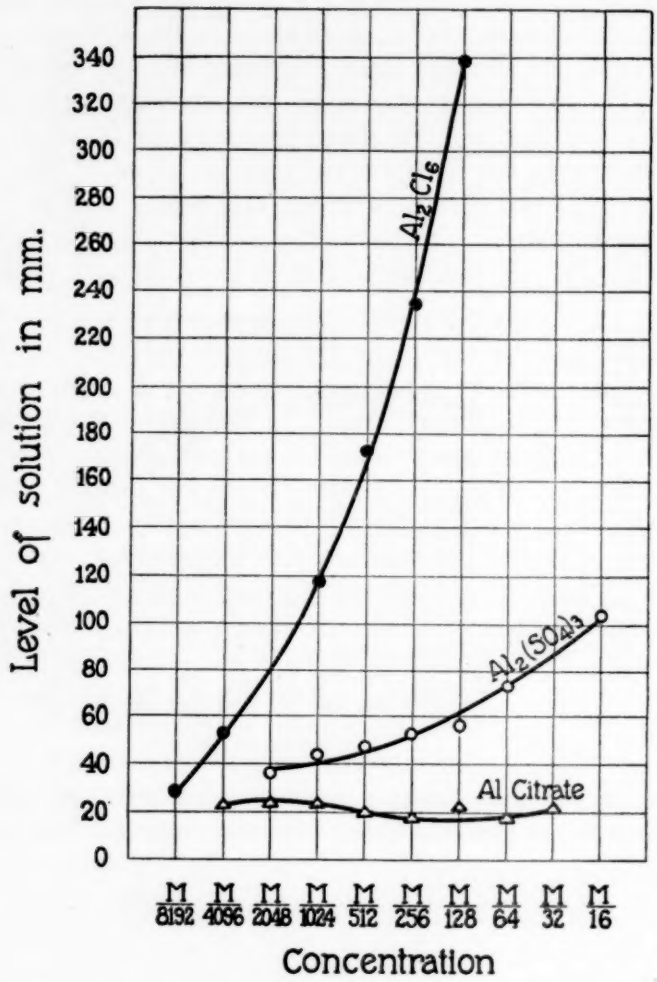

FIG. 13. Curves representing the attraction of different aluminium salts for negatively charged particles of water. The attraction is a maximum in the case of Al_2Cl_6, is less in the case of $Al_2(SO_4)_3$, and is practically lacking in the case of aluminium citrate, showing the increase in the repelling effect of the anion upon the negatively charged particles of water with increasing valency of the anion.

IV. Further Proof that the Negative Osmosis Is Due to the Repelling Action of the Ion with the Same Sign of Charge as that of the Electrified Water Particles.

When we fill the collodion flask with a M/256 solution of Na_2SO_4 (made neutral or slightly alkaline) and dip the flask into distilled water, we notice a rather high initial rate of diffusion of water into the flask caused by the fact that the attraction of the SO_4 ion with its two charges upon the positively charged particles of water is greater than the sum of the repelling action of the two Na ions. In higher concentrations of Na_2SO_4 this difference becomes less (Fig. 4), as we assume through the fact that for some reason the repelling action of the Na ions on the positively charged water particles increases more rapidly with further increase in concentration than the attractive action of SO_4. If this assumption is correct the addition of a salt of the type $MgCl_2$ to a M/256 solution of Na_2SO_4 should lower the rate of diffusion of water into the M/256 solution of Na_2SO_4 more than the addition of the same concentration of KCl; since the repelling effect of the bivalent Mg ion upon the positively charged water particles is greater than the repelling effect of the monovalent K ions. The addition of LiCl should lower the rate of diffusion of water more than the addition of KCl but less than $MgCl_2$, since the electrostatic effect of Li is greater than that of the K ion but less than that of the Mg ion. Fig. 14 shows that this is actually true. 100 cc. of M/256 Na_2SO_4 (pH about 6.0) contained 0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, and 25.6 cc. of M/4 KCl, LiCl, or $MgCl_2$. The addition of KCl caused only a slight diminution in the rate of diffusion when 12.8 to 25.6 cc. of M/4 KCl were added. LiCl caused more of a drop, but a slight addition of $MgCl_2$ caused a considerable drop, as our theory demands.

When, however, a salt with polyvalent anion and monovalent cation like $K_4Fe(CN)_6$ is added to M/256 Na_2SO_4 the attraction of the ferrocyanide ion for water counteracts to a large extent the drop caused by the weak repelling action of the K ions added. Fig. 3 shows that the attraction of the $Fe(CN)_6$ ion for water is greater than that of the sulfate ion. Hence the addition of slight quantities of $K_4Fe(CN)_6$ to M/256 Na_2SO_4 increases the initial rate of diffusion of water into the solution but as soon as the concentration of $K_4Fe(CN)_6$ added reaches

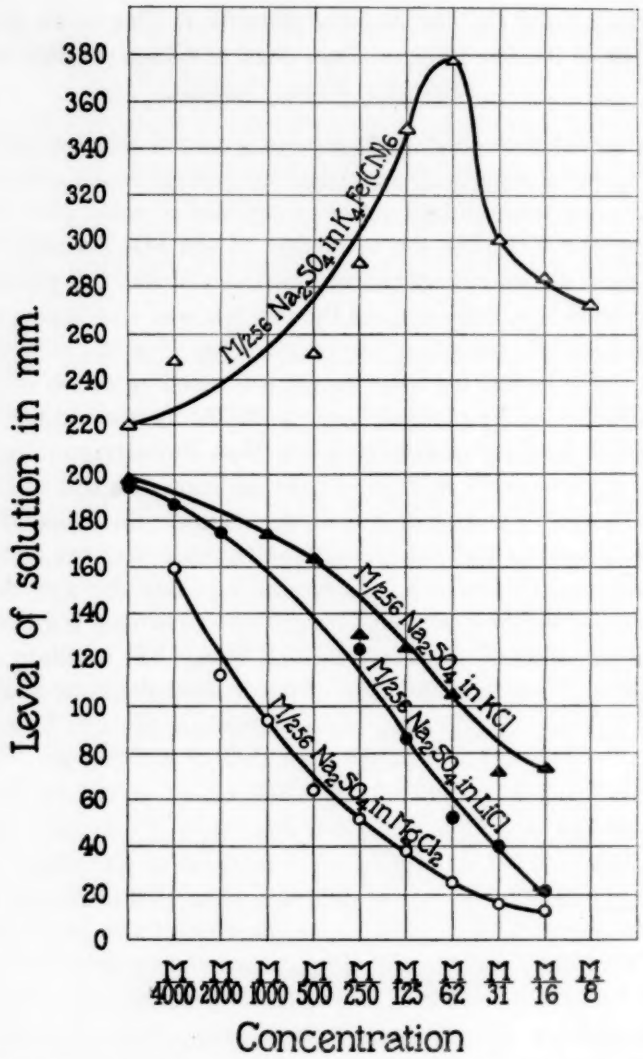

FIG. 14. Curves showing that when we add increasing concentrations of neutral salts with monovalent anion to a neutral M/256 solution of Na_2SO_4 the attraction of the Na_2SO_4 solution for water is diminished. Since this diminution increases in the order K, Li, Mg, the latter being the most depressing ion, it supports the idea that the drop observed in curves of Fig. 3 is due to the repelling action of the cation upon the positively charged particles of water.

the concentration of M/62 a further addition of $K_4Fe(CN)_6$ lowers the rate of diffusion of water into the solution. From this concentration on the repelling action of the K and Na ions upon water prevails over the attractive action of the anion.

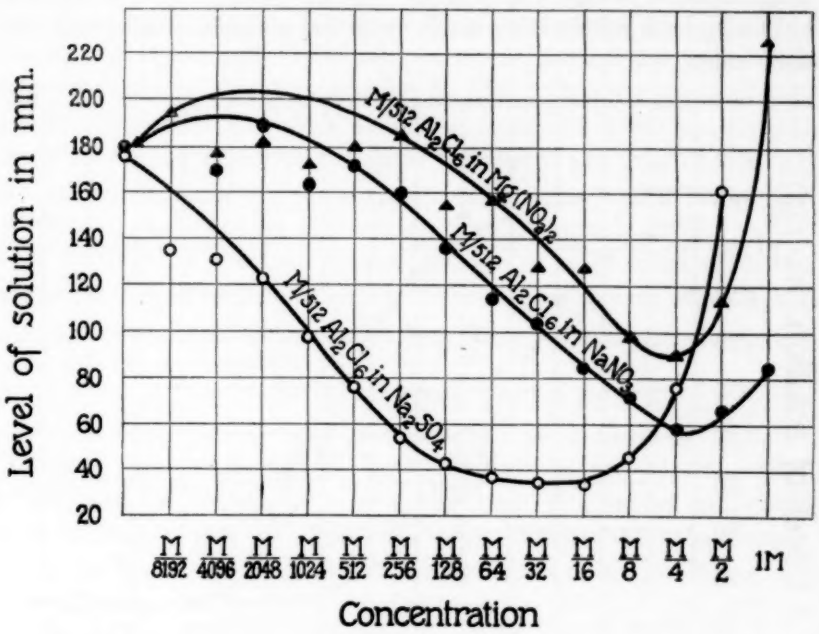

FIG. 15. Curves showing that when we add increasing concentrations of $NaNO_3$, $Mg(NO_3)_2$, and Na_2SO_4 to M/512 solutions of Al_2Cl_6 the depressing effect is greater for SO_4 than for NO_3. Since the water diffuses in this case in the form of negatively charged particles the curves support the idea that the depressing effect of the salt added is due to the repelling action of the anion upon the negatively charged particles of water.

When the collodion flask (previously bathed in gelatin) is filled with M/512 Al_2Cl_6 and dipped into a beaker with distilled water, water diffuses at first very rapidly into the flask. The water is in this case negatively charged and attracted by the Al ion and repelled by the anion. When we add small quantities of a salt with bivalent anion, *e.g.* Na_2SO_4, to the Al_2Cl_6 solution the attraction of the Al_2Cl_6 solution for water is diminished more rapidly than when we add a salt with a

monovalent anion like $NaNO_3$ or $Mg(NO_3)_2$ (Fig. 15). The Mg ion attracts negatively charged water more powerfully than the $NaNO_3$ and hence the depressing effect of $Mg(NO_3)_2$ is smaller than the depressing effect of $NaNO_3$. Fig. 16 shows that the depressing effect of the addition of H_2SO_4 upon M/512 Al_2Cl_6 is greater than the addition of HNO_3, both acids acting similarly to the potassium salts with the same anion.

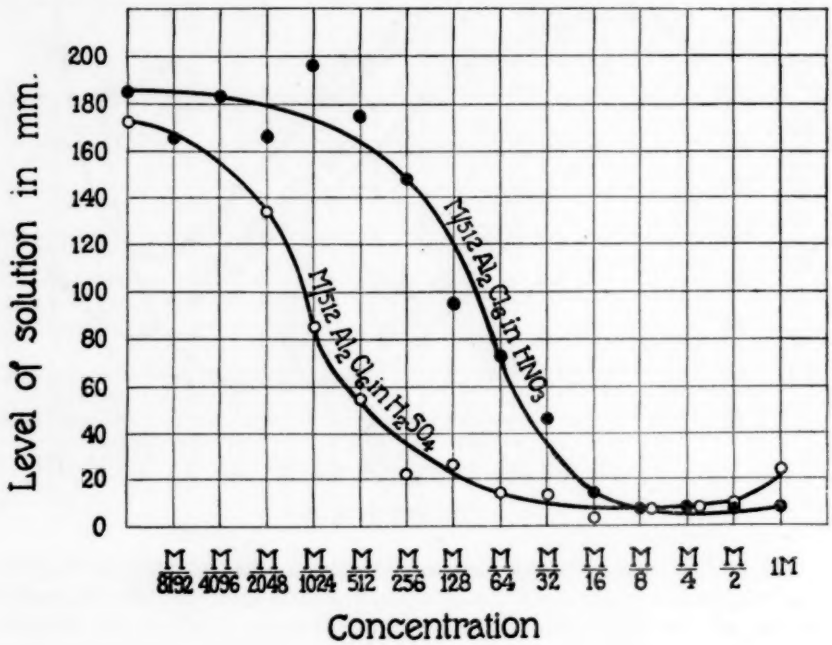

FIG. 16. Curves showing that the depressing effect of HNO_3 and H_2SO_4 is similar to the depressing effect of $NaNO_3$ and Na_2SO_4 when added to M/512 Al_2Cl_6.

All these facts support our assumption that the drop in the curves representing the influence of the concentration of the electrolyte upon the rate of diffusion of water through collodion membranes is due to the fact that beyond a certain concentration the repelling action of the ion with the same sign of charge as that of water increases more rapidly with a further increase in concentration than the attractive action of the ion with the opposite charge upon the water.

SUMMARY.

1. When a watery solution is separated from pure water by a collodion membrane, the initial rate of diffusion of water into the solution is influenced in an entirely different way by solutions of electrolytes and of non-electrolytes. Solutions of non-electrolytes, *e.g.* sugars, influence the initial rate of diffusion of water through the membrane approximately in direct proportion to their concentration, and this influence begins to show itself under the conditions of our experiments when the concentration of the sugar solution is above M/64 or M/32. We call this effect of the concentration of the solute on the initial rate of diffusion of water into the solution the gas pressure effect.

2. Solutions of electrolytes show the gas pressure effect upon the initial rate of diffusion also, but it commences at a somewhat higher concentration than M/64; namely, at M/16 or more (according to the nature of the electrolyte).

3. Solutions of electrolytes of a lower concentration than M/16 or M/8 have a specific influence on the initial rate of diffusion of water through a collodion membrane from pure solvent into solution which is not found in the case of the solutions of non-electrolytes and which is due to the fact that the particles of water diffuse in this case through the membrane in an electrified condition, the sign of the charge depending upon the nature of the electrolyte in solution, according to two rules given in a preceding paper.[1]

4. In these lower concentrations the curves representing the influence of the concentration of the electrolyte on the initial rate of diffusion of water into the solution rise at first steeply with an increase in the concentration, until a maximum is reached at a concentration of M/256 or above. A further increase in concentration causes a drop in the curve and this drop increases with a further increase of concentration until that concentration of the solute is reached in which the gas pressure effect begins to prevail; *i.e.*, above M/16. Within a range of concentrations between M/256 and M/16 or more (according to the nature of the electrolyte) we notice the reverse of what we should expect on the basis of van't Hoff's law; namely, that the attraction of a solution of an electrolyte for water diminishes with an increase in concentration.

5. We wish to make no definite assumption concerning the origin of the electrification of water and concerning the mechanism whereby ions influence the rate of diffusion of water particles through collodion membranes from pure solvent to solution. It will facilitate, however, the presentation of our results if it be permitted to present them in terms of attraction and repulsion of the charged particles of water by the ions. With this reservation we may say that in the lowest concentrations attraction of the electrified water particles by the ions with the opposite charge prevails over the repulsion of the electrified water particles by the ions with the same sign of charge as that of the water; while beyond a certain critical concentration the repelling action of the ion with the same sign of charge as that of the water particles upon the latter increases more rapidly with increasing concentration of the solute than the attractive action of the ion with the opposite charge.

6. It is shown that negative osmosis, *i.e.* the diminution of the volume of the solution of acids and of alkalies when separated by collodion membranes from pure water, occurs in the same range of concentrations in which the drop in the curves of neutral salts occurs, and that it is due to the same cause; namely, the repulsion of the electrified particles of water by the ion with the same sign of charge as that of the water. This conclusion is supported by the fact that negative osmosis becomes pronounced when the ion with the same sign of charge as that of the electrified particles of water carries more than one charge.